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TOXICOLOGY PROGRAM
Technology Branch
Communicable Disease Center
Atlanta, Ga. 30333

Spontaneous Malignant Gastric Tumor in a Rhesus Monkey *(Macaca mulatta)*

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THE OVERALL low incidence of spontaneous tumors in monkeys has been pointed out repeatedly.[1,2] As a rule, the induction of tumors with known carcinogens is also unsuccessful in monkeys.[1,2] Occasionally, tumors have been produced in monkeys with agents such as ionizing irradiation,[3] high boiling, catalytically cracked oil,[4] diesel lubricating oils,[5] and radium.[6,7]

Because of the apparent rarity of monkey tumors, a malignant tumor of the stomach that developed spontaneously in an old female Rhesus monkey (*Macaca mulatta*) is reported in this paper. The spontaneous tumors usually encountered at this site are polypoid lesions, referred to as benign papillomas, adenomas, or adenomatous polyps.[1-3] An adenocarcinoma of the stomach was, however, reported by Schmey,[8] and Ruch[9] and Jungherr[10] cited a carcinoma of the stomach that was observed by Kluver (unpublished). The carcinoma observed by Schmey[8] in a *Cercopithecus rufoviridis* was medullary and composed of solid groups of small pleomorphic cells. Numerous tumor nodules were present in the stomach measuring up to 2.5 cm in diameter; and most of the abdominal lymph nodes were involved in the tumor process.

The gastric polyps were usually related to nematodes (*Nochtia nochti*) and could be produced experimentally by introducing the worms into the wall of the stomach.[1,11,12]

Report of a Case

Clinical Findings.—Our laboratory obtained this adult female Rhesus monkey in 1953 from Montgomery, Ala, where she had been in a poliomyelitis study. The date of birth and further details of her early life could not be obtained. She lived in our laboratory for nine years and was used in various experiments. In early 1953, she was given Puritan chow instead of Purina chow for two months and responded with a weight loss of 420 gm. For one month at the end of 1953, the monkey was fed apples that had been heavily sprayed with DDT, 2,2-bis(*p*-chlorophenyl)1,1-trichloroethane, and Demeton, a mixture of 0,0-diethyl 0-[2-(ethylthio)ethyl] phosphorothiate and 0,0-diethyl S-[2-ethylthio)ethyl]phosphorothiate in a ratio of approximately 2 to 1. The amount of insecticides ingested by the monkey in this fashion cannot be determined from the available data, but she did not suffer any noticeable ill effects. Almost two years later, in the summer of 1955, a daily dose of 2.8 mg/kg Demeton was given in the diet for 28 days. From January 1956 until her death in April 1962, the animal received daily an average dosage of 5.54 mg/kg DDT in her diet. Starting in 1956, numerous fat biopsies were taken from the subcutaneous adipose tissue and the omentum, and one liver biopsy was performed, all under sodium pentobarbital anesthesia. On several occasions antibiotics were administered for a variety of undiagnosed systemic illnesses, and the animal was occasionally given a 5% alcohol solution. The peak weight of 8,870 gm was reached in 1960. After that, gradual weight loss set in, and the final weight was 5,160 grams. Six months prior to death the animal developed poor appetite. Shortly before death she had loose stools, swelling of the lower extremities, and finally ascites. During the week prior to death a paracentesis was performed on three separate occasions yielding 300, 150, and 150 ml of milky fluid, respectively.

Autopsy Findings.—The animal showed marked emaciation. A milky effusion was present in both pleural cavities. The lungs were congested and the diaphragm was fixed to the stomach.

The abdominal cavity contained a large amount of milky fluid. Numerous old adhesions were found throughout the abdomen. The etiology of these adhesions could not be determined with certainty; however, they might well be a result of the repeated abdominal surgery.

The stomach was dilated and a huge tumor was found within the region of the cardia just to the left of the esophageal opening. It infiltrated the

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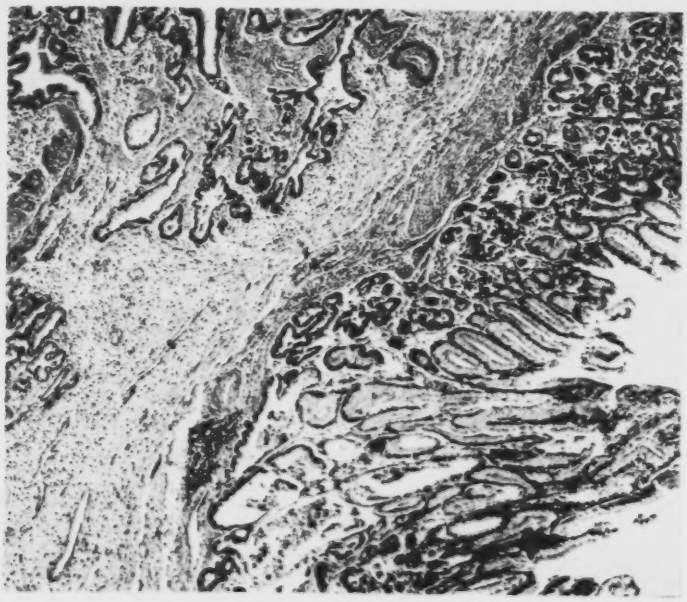

Fig 1.—Section of the tumor shows mucosa with well-differentiated glands penetrating the muscularis propria and lying in the submucosa (hematoxylin and eosin, ×40).

stomach wall and projected into the lumen. The esophagus was patent and admitted a finger easily. The tumor was grayish-white, firm, well circumscribed, and weighed 105 gm. Neither metastases nor parasites were observed.

No other gross findings of note were observed in any of the other organs. The brain was not examined. It was not stated in the autopsy protocol whether the thoracic duct was involved in the tumor process.

Results

Microscopic Description.—The tumor appeared to arise from the mucosa, immediately at the esophageal-cardiac junction, and it infiltrated downward through the wall of the stomach. Typical sections are shown in Fig 1-4. In some areas fairly well-differentiated glandular epithelium was bserved

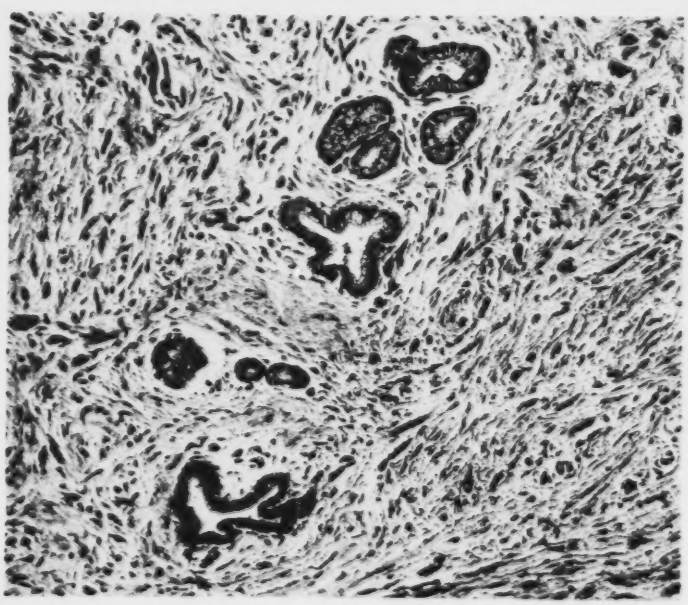

Fig 2.—This section demonstrates well-differentiated glandular elements in a deeper part of the tumor surrounded by spindle-shaped sarcomatous-appearing elements and collagen fibers (hematoxylin and eosin, ×135).

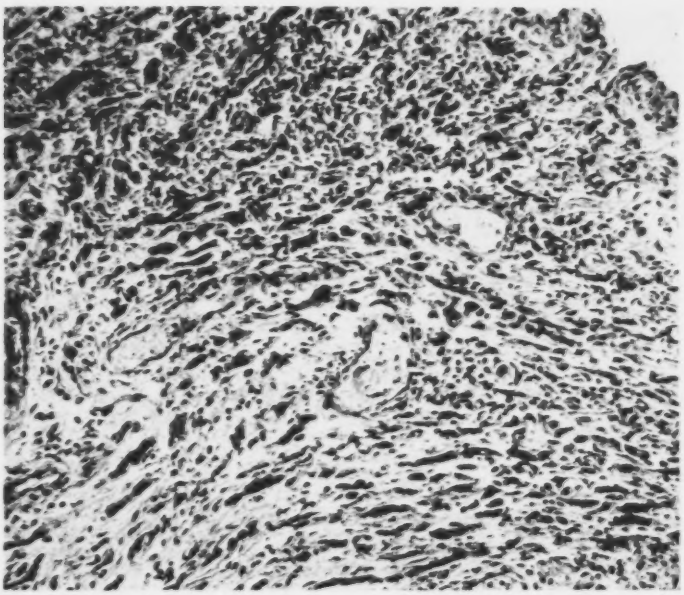

Fig 3.—In another section of the mucosa, irregular-shaped glands lined by atypical cells with vacuolated cytoplasm are shown. They are interspersed by spindle-shaped cells. Between these cells and around the glands, formation of collagen and reticulum fibers is seen (hematoxylin and eosin, × 250).

among wide areas of sarcomatous-appearing elements with spindle-shaped nuclei of more or less uniform size, which were surrounded by filaments of pink-staining cytoplasm. In addition, plumper cells resembling anaplastic adenocarcinoma cells, often with very bizarre shapes, were observed at intervals. They occurred singly and in small groups. The cytoplasm of these cells was more abundant, highly eosinophilic, and at times foamy or occasionally coarsely vacuolated. These cells formed acini in some sections. Their vacuolated cytoplasm suggested mucus production. A mucicarmine stain and periodic acid-Schiff stain performed on several sections containing these cells were negative, while positive-staining material was present in the epithelial cells of the better differ-

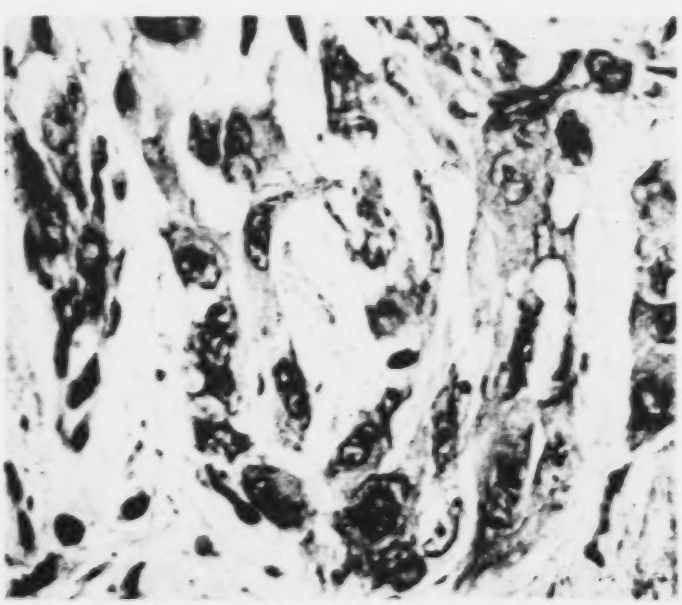

Fig 4.—High magnification of an aggregate of plump and sipndle-haped cells. Note prominent nucleoli and cellular pleomorphism (hematoxylin and eosin, × 610).

entiated glands. Masson's trichrome stain showed sparse strands of blue-staining collagen fibers surrounding the gland and acini. The individual sarcomatous-appearing cells were also separated from each other by a scanty number of similarly staining fibers. In a few sections collagen was more abundant, and active stromal proliferation was observed in most sections. A reticulum stain showed a meshwork of fine argentaphil fibers surrounding individual spindle-shaped tumor cells or groups of tumor cells.

Most of the cells described above had criteria of malignant cells such as pleomorphism, clumped chromatin, very large nucleoli, and bizarre form. Numerous mitotic figures were also observed. The well-differentiated glandular cells shown in Fig 1 and 2 represented an exception. Infiltration of the wall of the stomach was observed grossly as well as microscopically but neither invasion of surrounding structures nor metastases were found at autopsy, which is unusual for a malignant tumor of this size.

The microscopic examination of other organs did not reveal anything of note except a mild chronic interstitial nephritis and a mild chronic pericholangitis.

Conclusions

A microscopically unusual, malignant neoplasm of the stomach that had developed spontaneously in an old female Rhesus monkey is reported. It was concluded that this tumor represented a carcinoma in which highly anaplastic cellular elements were intermixed with well-differentiated adenocarcinoma cells. A great deal of stromal proliferation was also observed in many areas of the tumor, such as is characteristic for a scirrhous carcinoma. The neoplasm had invaded the stomach wall and the tumor cells showed all criteria of malignancy, but metastases were not found, which is unusual for a tumor this size. It was felt that the neoplasm had developed spontaneously since there is no evidence that exposures to poliomyelitis or pesticides influenced the development of cancer in this monkey. No other cases have appeared in the colony which has been maintained for 15 years. In addition to the monkey that developed cancer, we have held 18 others for 7-15 years after they were retired from poliomyelitis studies. Five other monkeys out of the colony were fed DDT for the same or longer periods and at the same or higher dosages as the monkey reported in this paper (see preliminary report by Durham et al[13]). The studies on Demeton were brief and involved only eight animals.

Dr. Harold L. Stewart and Dr. Catherine C. Snell from the National Cancer Institute, Bethesda, Md, provided guidance for this study.

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Seasonal Neonatal Hyperbilirubinemia

Neonatal hyperbilirubinemia, more commonly referred to as neonatal jaundice, is a common finding in hospital nurseries. While many etiologic factors have been identified, seasonal variations in the incidence of neonatal hyperbilirubinemia have not, to the author's knowledge, been described.

During the last quarter of 1965, the occurrence of an excess number of cases of neonatal hyperbilirubinemia in the nursery of a hospital in a small California community was suspected by local physicians. They had eliminated, to their satisfaction, the possibility of laboratory error and had considered and rejected a number of well-known causal factors such as prematurity, sepsis, blood group incompatibility, maternal drug use during pregnancy, and therapeutic agents administered to the newborn during the first days of life. They had also excluded a number of possible genetic and metabolic causes. These physicians then reviewed an exhaustive list of compounds used in the hospital for cleaning, disinfecting, insect control, and other general maintenance work to

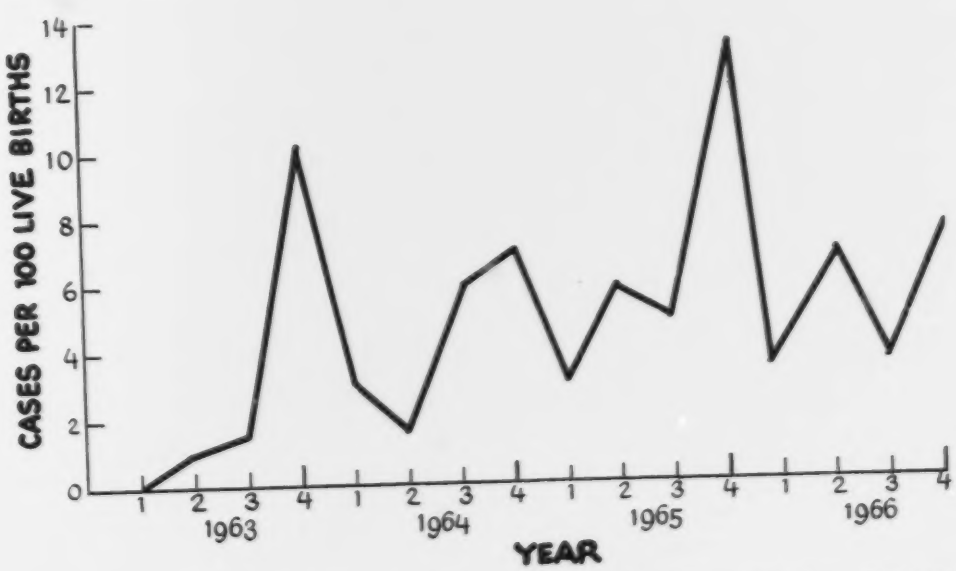

FIG. 1. Cases of neonatal hyperbilirubinemia per hundred live births by quarter. Hospital one, 1963-1966. (Source: California State Department of Public Health-Bureau of Occupational Health.)

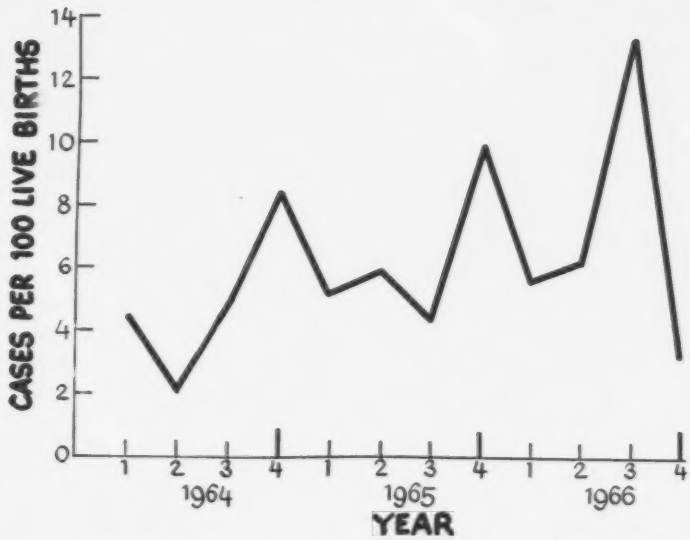

FIG. 2. Cases of neonatal hyperbilirubinemia per hundred live births by quarter. Hospital two, 1964-1966. (Source: California State Department of Public Health-Bureau of Occupational Health.)

determine whether any of these materials might be causally involved. They found no apparent association. Because the area is one in which agriculture is carried out on an intensive basis, they next considered the possibility that agricultural chemicals might in some way be involved. Defoliating agents, which were applied heavily during the latter months of each year to cotton grown in fields adjacent to the community, were of particular interest. At this time, because of the increasing complexity of the problem, the situation was brought to the attention of the California Department of Public Health.

METHODS

The first task undertaken, and that reported here, was to establish the existence of a true seasonal variation in neonatal jaundice in the community hospital. This problem was approached through a retrospective analysis of the hospital records of all newborns delivered at the community hospital during the 4-year period, 1963–1966. A similar, but less detailed, analysis for the years 1964–1966 was undertaken at a second hospital situated in a community 15 miles away. This second community, like the first, was small in size and located in the same predominantly agricultural setting. Furthermore, both hospitals provided a generally similar, high level of medical care, and, in both, attending physicians delivered from 350 to 450 infants per year. It was hoped that a review of records in this second community hospital would help determine whether any observed seasonal variation in neonatal jaundice was related to an event acting on the entire area or, insofar as could be determined, acting only in a single hospital. (The hospital of primary interest is designated hospital one and the second as hospital two.)

A case of neonatal hyperbilirubinemia was defined as an infant whose highest recorded indirect reacting serum bilirubin level was equal to or greater than 10 mg/100 ml. In some instances, only one recording was available; therefore, peak levels were often missed. Not included in this group were those infants whose hyperbilirubinemia was clearly due to hemolytic disease of the newborn as proven by a positive direct Coombs test.

During the period under review, bilirubin levels had been measured only when the attending physician felt jaundice was in excess of

physiologic levels. Therefore, the visible presence of more than physiological jaundice and a confirmatory test are implicit in the case definition.

At each of the two hospitals, the records of all newborns delivered during the years of interest were reviewed. Case cards were completed for all those meeting the case definition. For comparison purposes, a systematic sample of all newborn records was obtained from hospital one by selecting every sixth birth by month for each of the years which were studied. No comparison data were obtained from hospital two.

The following information, where available, was abstracted from each case and comparison record: name of attending obstetrician and pediatrician, name of child, birth date, sex, birth weight, medical history of the pregnancy, length of hospital stay, method of feeding, laboratory determinations of ABO and Rh blood type, Coombs test, hemoglobin, and serum bilirubin levels; mother's name, address, age, race, ethnic background (Spanish or other surname), and history of previous pregnancies; and father's name, address, age, race, employment, and ethnic background (Spanish or other surname).

Results

The Clinical Picture: The typical clinical picture was of a healthy infant who became visibly jaundiced on the third or fourth day of life. Laboratory studies revealed a mild to moderate nonhemolytic unconjugated hyperbilirubinemia which cleared without treatment on the fifth or sixth day of life. Generally the babies thrived and were without other manifestations of disease. In a rare case, ABO incompatibility was suspected. There were a few instances in which infection or cephalohematoma was present and may have contributed to the hyperbilirubinemia. These recognizable conditions occurred sporadically over the 4-year study period and did not contribute in any systematic way to the quarterly incidence of hyperbilirubinemia.

Distribution of Cases: Figures 1 and 2 show the distribution of hospital one cases by quarters for the years 1963–1966 and hospital two cases for the years 1964–1966.

The highest quarterly incidence of neonatal jaundice in hospital one occurred during the fourth quarter of each of the 4 years under observation (Fig. 1). Moreover, when the 4 years are grouped by quarter (Table I), the fourth quarter surplus of cases is highly significant statistically. No such significance can be attributed to the quarterly variations in neonatal jaundice in hospital two (Table I). However, even though not significant statistically (Table I), there is a clear tendency toward a fourth quarter predominance in 2 of the 3 years for

TABLE I

Observed Versus Expected Distribution of Cases of Neonatal Hyperbilirubinemia by Quarter of Year of Birth

Hospital	*Year*	*Quarter*	*Total Live Births*	*Cases*		*P*
				OBS	*Exp*	
One	1963–1966	1	504	13	25.7	
		2	446	17	22.7	
		3	556	23	28.4	
		4	554	52	28.2	
Total			2,060	105		<.01
Two	1964–1966	1	227	12	14.5	
		2	251	12	16.0	
		3	273	21	17.4	
		4	285	21	18.1	>.5*
Total			1,036	66		

* Not significant.
OBS = observed; Exp = expected.

TABLE II

Method of Feeding Among Case and Comparison Newborns by Quarter of Year of Birth Hospital One, 1963–1966

Quarter	*Feeding Method*	*Cases*	*Comparison Sample*	*P*
Total	Breast	33	147	
	Formula	72	194	<.05
1	Breast	6	34	N.S.
	Formula	7	49	
2	Breast	5	34	
	Formula	12	40	N.S.
3	Breast	8	36	
	Formula	15	58	N.S.
4	Breast	14	43	
	Formula	38	47	<.02

N.S.—not significant.

which data are available from this second hospital (Fig. 2).

Prematurity: Because, in general, premature infants are known to have higher bilirubin levels than full-term infants,[1] this variable was given close attention. Due to the retrospective nature of the study, a precise definition of prematurity incorporating the true length of gestation could not be realistically applied. Prematurity, therefore, is simply defined here as a birth weight of less than 5 lb. While 6 of the 19 premature babies in our sample had elevated bilirubin levels compared to 30 out of 470 babies not so classified, the fourth quarter excess was mostly among non-premature infants, so the phenomenon was not explained by prematurity.

Sex: Elevated bilirubin levels were more frequent among males than females, but in the fourth quarter an equal number of both sexes had such elevations.

Race and Ethnic Background: Limitations inherent in a record study of this type do not permit a precise analysis of either the race or ethnic background of the families involved. The only three rubrics which were consistently recorded by hospital personnel and physicians to describe "race" were white, Negro, and oriental. These three classifications did not seem to be associated with the elevated bilirubin levels.

Method of Feeding: Reports indicate that bilirubin levels among some breast-fed infants may be higher than bilirubin levels among those fed in other ways.[2-4] For this reason, feeding data were abstracted from both case and comparison records from hospital one. As a general rule, both breast- and formula-fed infants received supplemental water intermittently on the second and subsequent days of life. Moreover, in many instances breast feeding was accompanied by formula supplementation. For the purposes of this study, an infant who received any portion of his feeding from the breast, regardless of formula supplementation, was considered breast-fed.

Table II summarizes method of feeding among the infants with elevated bilirubin levels (cases) and those in the comparison sample. All data are from hospital one. During the fourth quarter, this comparison suggests a strong association ($p < .02$) between hyperbilirubinemia and formula feeding. A similar association appears when all four quarters are grouped, but it is not evident during the first, second, or third quarters. The possible significance of this observation will be discussed.

Discussion

The goals of this study were threefold: (1) to verify the existence of an annual fourth quarter excess of neonatal jaundice, (2) to establish causality, and (3) based on a knowledge of causality, to devise and implement preventive techniques.

Thus far we have verified to our satisfaction the existence of an annual fourth quarter excess of neonatal jaundice in hospital one. It seems likely to us, moreover, that a similar excess has occurred on occasion in hospital two.

The question of causation remains relatively obscure. We have been unable to find reference to publications by others which indicate that neonatal bilirubin levels have been observed elsewhere to fluctuate in any systemic manner with season or time of year. Moreover, the usual etiologic factors known to be associated with increased bilirubin levels have been excluded, insofar as possible. Faced with these observations, we have been led to think in terms of a causal agent, more likely chemical than microbial, acting on a seasonal basis. This approach has prompted a consideration of three questions. First, which chemical agents might be expected to appear in the community on a seasonal basis, especially during the fourth quarter of the year? Second, how might such a agent gain access to the child, either *in utero* or during the first days of life. Third, which, if any, agent so identified would be capable of affecting neonatal bilirubin levels.

Additional studies are underway to see if an agent can be identified. Predominance of the finding in formula-fed infants suggests that the water used may transmit some agent to which the newborn is unusually susceptible.

We are currently engaged in a 1-year prospective study of neonatal bilirubin levels among infants born in the two hospitals of interest. Bilirubin and hematocrit levels are being determined on all infants whether visibly jaundiced or not. The prenatal course of all prospective mothers scheduled for confinement in either of the two hospitals is being followed with the cooperation of community physicians. Following delivery, all relevant hospital records are abstracted. Mothers are interviewed 4 to 6 weeks postpartum, and information on the

baby's progress is recorded. Simultaneously, agricultural chemical usage data are being collected and analyzed. The results of this study will be reported later.

Summary

A seasonal variation in the incidence of neonatal nonhemolytic, unconjugated hyperbilirubinemia has been observed in a small, predominantly agricultural community. A total of 3,096 records, representing all newborns delivered during a 4-year period (1963–1966) in one local hospital and during an overlapping 3-year period (1964–1966) in another, were reviewed. A case was defined as an infant whose highest recorded unconjugated bilirubin level reached 10 mg/100 ml during the first days of life. Infants with clear-cut hemolytic disease of the newborn were excluded from consideration. One hundred seventy cases were identified. In one hospital, an excess of cases occurred during the fourth quarter of each of the 4 years reviewed. A similar trend was apparent in the second hospital during 2 of the 3 years reviewed.

The cause of this systematic fluctuation is unclear. Insofar as possible, factors commonly associated with neonatal hyperbilirubinemia were excluded. During the peak incidence periods, a surplus of cases among infants fed with tap water-containing formula was noted.

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J.E.M. is a medical student, Vanderbilt University School of Medicine. T.S.F. is a medical student, Harvard University School of Medicine.

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Determination of Cholinesterase by an Automated pH Stat Method

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THE INCREASED use of organic phosphorus insecticides has necessitated the development of more reliable methods for the detection of exposure to these materials. Since the most important pharmacologic effect of these compounds is inhibition of cholinesterase, the level of activity of this enzyme is often used to establish possible exposure. Some of the compounds are potent anticholinesterases per se, while others are converted within the plant or animal body into compounds having this activity. Various means of measuring cholinesterase have been discussed in an excellent review by Witter.[1] Most of the methods are satisfactory for measurement of cholinesterase activity in human blood—a tissue which has a relatively large content of the enzyme. However, several commonly used laboratory animal species have comparatively low blood cholinesterase content, and its precise measurement requires a method with high sensitivity. Although the potentiometric method[2] has been modified for use with several animal species,[3] it is still somewhat lacking in sensitivity and precision. Even though numerous cholinesterase determinations have been made using animal blood, there are few detailed studies of the methods of analysis for various animal species. An exception is the detailed study of the manometric method for rats made by Witter.[4]

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The technique of continuous titration of the acetic acid released from acetylcholine by the enzyme cholinesterase, as introduced by Stedman et al[5] and modified by Glick[6] and Delaunois and Casier,[7,8] overcomes many of the undesirable features of other methods. This method does not utilize buffers, is temperature and atmospherically controlled, has easily calculated units which are interconvertible with most other methods, and is rapid and simple to run. In addition, the substrate and enzyme concentrations can be adjusted and maintained at levels which allow optimal enzyme activity. The use of a commercially available instrument for the automatic titration and recording of the enzyme activity has been described by Jensen-Holm et al[9] and Jorgensen.[10] A laboratory fabricated instrument capable of essentially the same determination was reported by Delaunois.[11] Although the titrimetric procedure utilizing a commercially available automatic titrator has been in use for cholinesterase determination in human blood for several years in the Toxicology Laboratory of the National Communicable Disease Center and has proved to be highly satisfactory, a description of the method, which we consider superior to those described earlier,[9-11] has not been published. Furthermore, modifications suitable for use with blood from different species of laboratory animals have not been published. This report describes the method used and some kinetics of the cholinesterase determination in the dog, rabbit, rat, and man. The method provides a well defined and precise measurement of the effects of organophosphate insecticides upon these species, which heretofore could be estimated only approximately in toxicological experiments.

Materials and Methods

All reagents unless otherwise indicated were analytical grade. Acetylcholine perchlorate, 0.110M, was made by dissolving 0.675 gm of crystalline substrate in distilled water and diluting it to 25 ml in a volumetric flask. The sodium choloride solution, 0.154M, (0.9%) was made by dissolving 9 gm in distilled water in a 1-liter volumetric flask. Sodium hydroxide, 0.0100N, was made by diluting an appropriate amount of approximately 1N sodium hydroxide which had been accurately standardized by titration with 0.1N potassium acid phthalate (primary standard) using phenophthalein as indicator. Ten milliliters of 0.0100N sodium hydroxide was diluted to 50.00 ml with distilled water in a volumetric flask to obtain a concentration of 0.0020N sodium hydroxide.

Collection of Blood.—Blood was obtained from the rat by cardiac puncture, from the rabbit by medial ear artery puncture, from the dog by venipuncture of the foreleg, and from humans by venipuncture of a anticubital vein. The blood was drawn into syringes rinsed with heparinized saline and transferred into a 2.5-ml glass-stoppered conical centrifuge tube (graduated to 0.05 ml) wetted with a small amount of additional heparin solution. The heparinized blood was immediately immersed in an ice bath. After centrifugation at 2,000 rpm for 15 minutes at 5 C, the plasma was removed and transferred to a labeled tube for storage or for immediate analysis of cholinesterase activity. The white blood cells were removed from the red blood cells (RBCs) by aspiration and about 0.5 to 1 ml of the packed RBCs were transferred to a 5-ml graduated conical centrifuge tube. Routinely, enough saline was added to bring the volume of the mixture to 3 ml. The cells were resuspended by gentle agitation with a beechwood applicator stick, and the tube was stoppered. After centrifuging as before, the washing was repeated and the cells centrifuged a third time. The saline from the final wash was removed, and the cells were hemolyzed with nine volumes of distilled water. The 10% RBC hemolysate was allowed to stand for at least ten minutes before assay.

All of the assays were performed with a pH stat apparatus consisting of: (1) a titrator with scale expander, (2) a titrigraph equipped with a 0.5-ml syringe and micrometer, and (3) a thermostated microtitration assembly heated by a circulating water bath.

RBC Cholinesterase Determination.—The determination of RBC cholinesterase was carried out at pH 8.00 at 37 C in a total volume of 5.0 ± 0.1 ml. Two milliliters of the 10% hemolysate of rat, dog, or rabbit cells or 0.5 ml of human RBC hemolysate were pipetted into the titration vessel; then, enough 0.15M saline solution was added to bring the volume to 4.8 ml. Nitrogen was allowed to blow into the vessel near the liquid surface during the subsequent steps. The instrument was allowed to titrate the mixture with 0.0100N sodium hydroxide to pH 8.00, the endpoint which was present on the instrument. (If the pH of the mixture was initially below 7.8, a 0.1N sodium hydroxide solution was added manually until the pH was almost 8, and then the instrument was allowed to titrate to exactly pH 8.00.) The total volume of base added in the preliminary titration was kept below 0.1 ml. In the case of hemolyzed rat blood, a preliminary instrumental titration of about three minutes was necessary in order to dissolve an unidentified crystalline material which occurred and to stabilize the instrument. The crystalline material developed only in the rat RBC hemolysate; it was readily dissolved by the addition of base to the mixture. When no further addition of base occurred and the instrument was stabilized, 0.1 ml of 0.11M acetylcholine perchlorate was added to the solution from a microliter syringe. The addition often caused the pH to increase a small amount above pH 8.00. The titrigraph was switched to "on" when the valve relay clicked at the first indication of slight decrease of pH below 8.00. The instrument was allowed to titrate the reaction for three minutes or until at least three minutes of the recording was linear.

Plasma Cholinesterase Determination.—The determination of plasma cholinesterase activity was essentially the same as for the RBCs except that adjustments in the quantity of plasma from the various species were necessary in order to compensate for wide variations in their activity.

One-half milliliter of dog or rabbit plasma or 0.15 ml of human plasma was used. The dog or human plasma and enough 0.15M saline to provide a total volume of 4.3 ml were pipetted into the thermostated reaction vessel. For the rabbit the plasma and enough 0.15M saline to bring the total volume to 4.75 ml were placed in the vessel. After adjustment of the pH to 8.00 and gassing with nitrogen, 0.6 ml of 0.11M acetylcholine perchlorate was deposited into the mixture for dog or human plasma and 0.15 ml of 0.11M substrate for the rabbit. The titration was then carried out in the same way as for the RBCs.

The determination of rat plasma cholinesterase was scaled down in order that only submilliliter blood samples would be needed. In this case, 0.1 ml of plasma was added to 2.4 ml

of 0.15M saline. After adjustment to pH 8.00 and gassing with nitrogen, 0.075 ml of 0.11M substrate was added from a microliter syringe and the titration carried out using 0.002N sodium hydroxide instead of the 0.01N sodium hydroxide used for all other determinations.

The calculations for determining the activity were made as follows: The instrument was calibrated in such a way that a deflection of 1.0 unit on the recorder represented an addition of 0.005 ml of titrant from the syringe.

$$\left.\begin{array}{l}\text{Micromoles of acetylcholine}\\ \text{hydrolyzed per minute per milliliter}\\ \text{of plasma or packed RBCs}\end{array}\right\} = \frac{A \times B \times N \times 1{,}000}{C \times D}$$

where A indicates the number of recorder units over period of titration; B, milliliters delivered by syringe per recorder unit; N, normality of titrant; C, time of titration in minutes; and D, milliliters of plasma or packed RBCs.

Results

Results With Various Species.—The Table shows the results of the determination of RBC and plasma cholinesterase of normal male dogs, rats, rabbits, and humans by the method herein described. The values obtained are expressed as micromoles of acetycholine hydrolyzed per minute per milliliter of plasma or packed RBCs. These values can be easily compared and are interconvertible with those of most other methods except where methods are based upon a change in pH over a preset time period, eg, the Michel method.[2]

Relation Between Substrate Concentration and Enzyme Activity.—Fig 1 presents data for the effects of substrate concentration upon ezyme activity for the four species. Increasing levels of substrate were added to the reaction vessel at the expense of saline, thus keeping the total volume of reaction mixture constant. The plasma cholinesterase activities of the dog, rat, and man were not inhibited by excesses of substrate. However, the rabbit plasma enzyme reached maximal activity at 1.3×10^{-3} M acetylcholine and then decreased steadily with increasing substrate. This phenomenon is characteristic of true cholinesterase which is found primarily in RBCs and certain other tissues. It has been shown by the use of specific substrates[12-14] that the cholinesterase activity of rat plasma involves both true and pseudocholinesterase. Presumably, the two enzymes occur in the plasma of other species as well. Studies of rabbit plasma in this laboratory with specific substrates have indicated a high activity for acetylmethylcholine and relatively low activity with either butyrylcholine or benzoylcholine, thus suggesting a relatively greater abundance of true than of pseudocholinesterase in this tissue. The substrate concentration chosen for the assay of dog, rat, and human plasma cholinesterase was 0.013M which provided a level well into the region of the activity curve approaching zero order kinetics where activity is no longer increased with increasing substrate. The maximal rate of hydrolysis by human plasma cholinesterase occurred at 0.02M acetylcholine with the manometric method.[15] The lower substrate concentration of 2.2×10^{-3} M was chosen for use with rabbit plasma to minimize substrate inhibition. This level was purposely slightly inhibitory. A slight excess was chosen because, as the reaction progresses and acetylcholine is hydrolyzed, its concentration is lowered and the optimal substrate concentration is approached. In all cases for all species, no more than 5% to 10% of the added substrate was hydrolyzed during the period of titration, thus always assuring the presence of an abundant supply of substrate.

Normal Cholinesterase Values ad Determined by the Titrimetric Method and Expressed in Micromoles of Acetylcholine Hydrolyzed per Minute per Milliliter of Plasma or Packed RBCs

Species	No. of Samples	Range	Mean ± SE
Dog			
RBC	3	1.5-1.8	1.7 ± 0.11
Plasma	3	1.7-1.9	1.8 ± 0.06
Rabbit			
RBC	28	0.50-2.8	2.1 ± 0.18
Plasma	28	0.48-1.2	0.76 ± 0.03
Rat			
RBC	42	1.0-2.8	1.7 ± 0.07
Plasma	42	0.30-1.2	0.62 ± 0.04
Man			
RBC	18	11.1-16.0	13.2 ± 0.31
Plasma	18	3.6-6.8	4.9 ± 0.02

The optimal substrate concentration for the RBC enzymes was 1.76×10^{-3}M for the dog, rat, and man and 0.88×10^{-3}M for the rabbit (Fig 2). The substrate concentration chosen for the RBC assay was 2.2×10^{-3}M for all species. This slightly inhibitory level was chosen for the same reason indicated

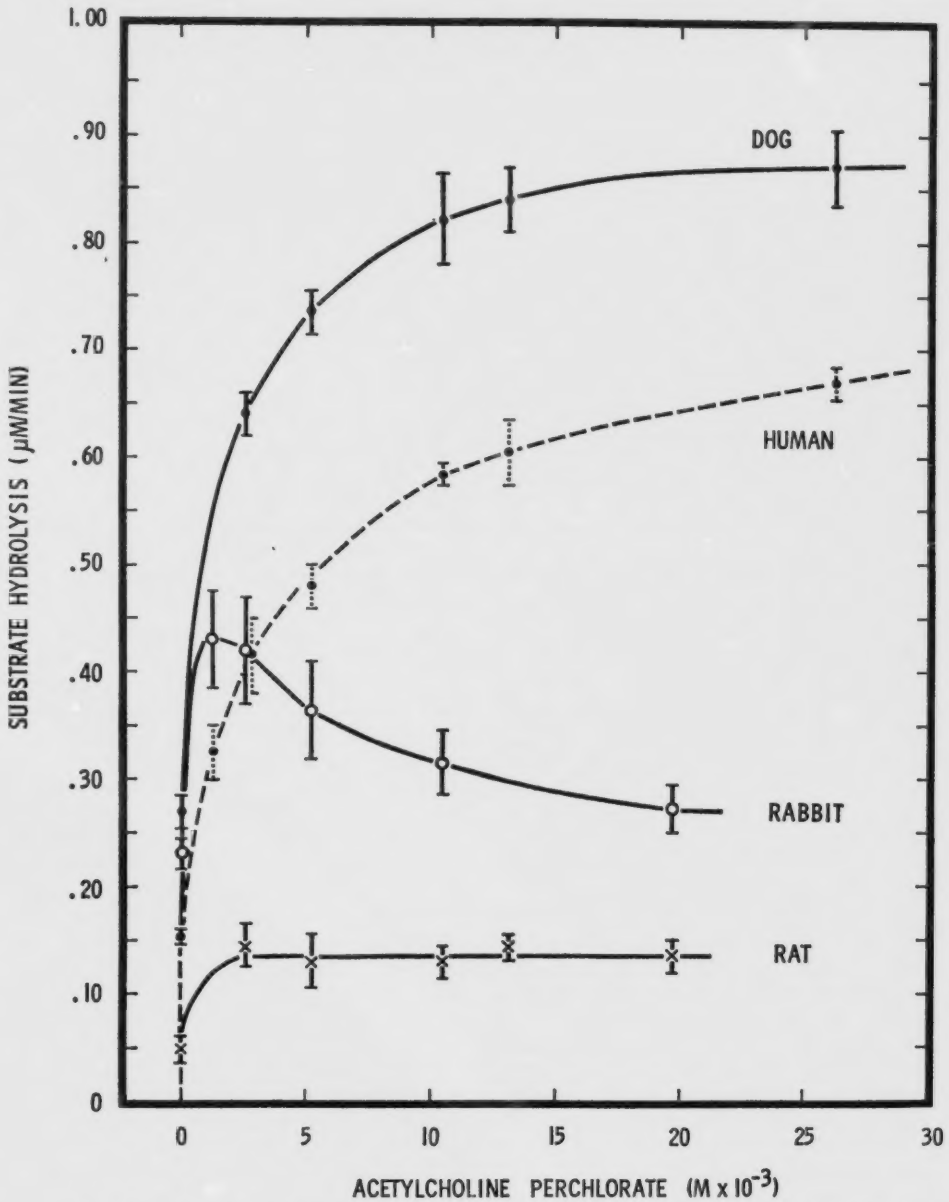

Fig 1.—Plasma cholinesterase activity as a function of substrate concentration. The hydrolysis of acetylcholine perchlorate was determined as described in the text. The volume of human plasma was 0.15 ml; the volume for other species was 0.5 ml.

above in conection with rabbit plasma. Alles and Hawes[15] found that maximal hydrolysis occurred at 3×10^{-3}M acetylcholine for human RBCs.

Relation Between Plasma and RBC Concentration and Enzyme Activity.—Using the standardized substrate levels discussed above, the effect on enzyme activity of vary-

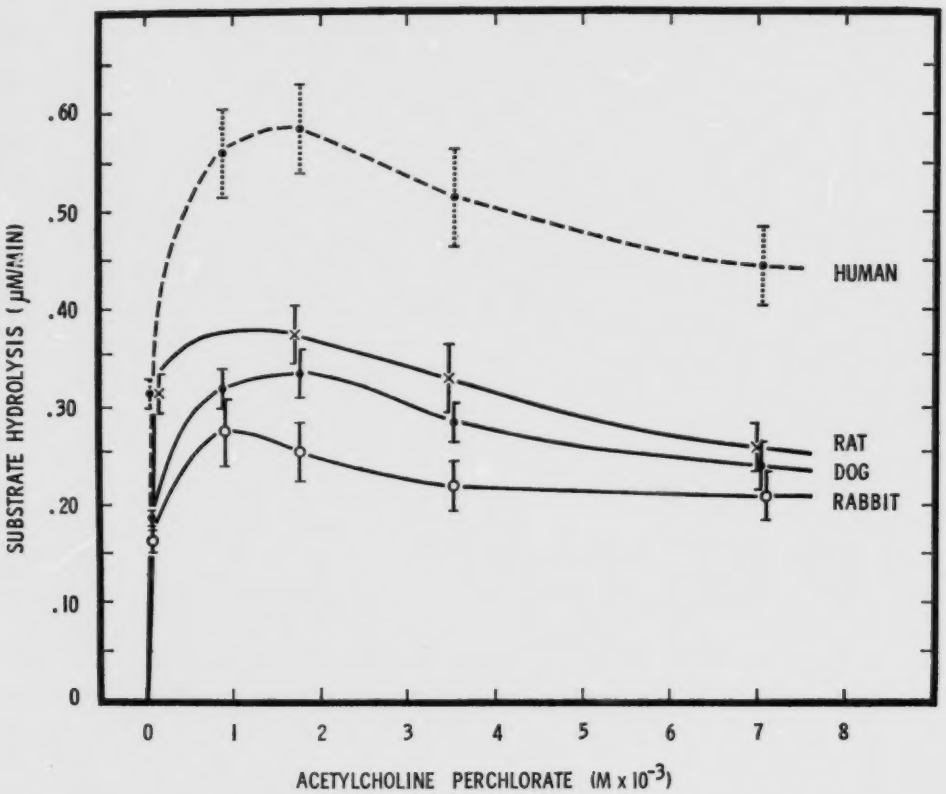

Fig 2.—Erythrocyte acetylcholinesterase activity as a function of substrate concentration. The hemolysate volume for all species was 0.1 ml. The conditions of assay are described in the text.

ing the amount of plasma or hemolyzed RBCs used per determination was studied. In the studies represented by Fig 3 and 4, the plasma or RBC hemolystate volume was increased at the expense of saline in the reaction vessel, while the total volume of reaction mixture was held constant. The variation in enzyme activity was found to be linear with increasing plasma concentrations for all four species used. The slopes were different because of inherent differences in enzyme activity among the species. All of the curves passed through the origin, except the one for the dog. The reason for the difference noted with the dog was not determined; however, it did not appear to be due to nonenzymatic hydrolysis. Nonenzymatic hydrolysis did not exceed 0.050/μmol/min in any instance. Since this rate is small in comparison with the activities generally measured, a correction was not applied. The activity also increased linearly with increasing RBC concentration for all species. At high levels of RBCs for the rat and dog, the curves changed slope. The reason for this inflection is not obvious, but it has been noted that rat erythrocyte acetylcholinesterase exhibits the same inflection when measured by the manometric method also.[4] In most instances the inflection occurs at enzyme activities greater than normal, and in the usual range the curve is linear.

All hemolysates were prepared as tenfold dilutions with water. Hemolysis was complete within ten minutes. Since the present procedure utilizes relatively high aqueous dilutions of erythrocytes, it was not necessary to add saponin to aid hemolysis, as was done by Michel.[2] The rat RBCs behaved differently from those of the other species.

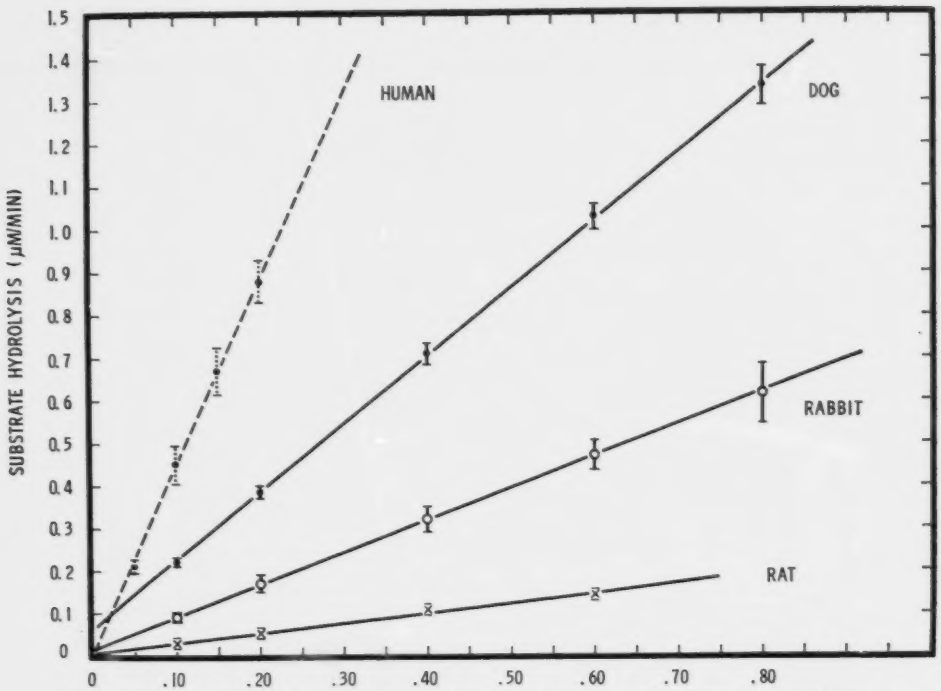

Fig 3.—Plasma cholinesterase activity as a function of enzyme concentration. The conditions of assay were the same as those for Fig 1, except that the substrate concentration was 6.6×10^{-5} M.

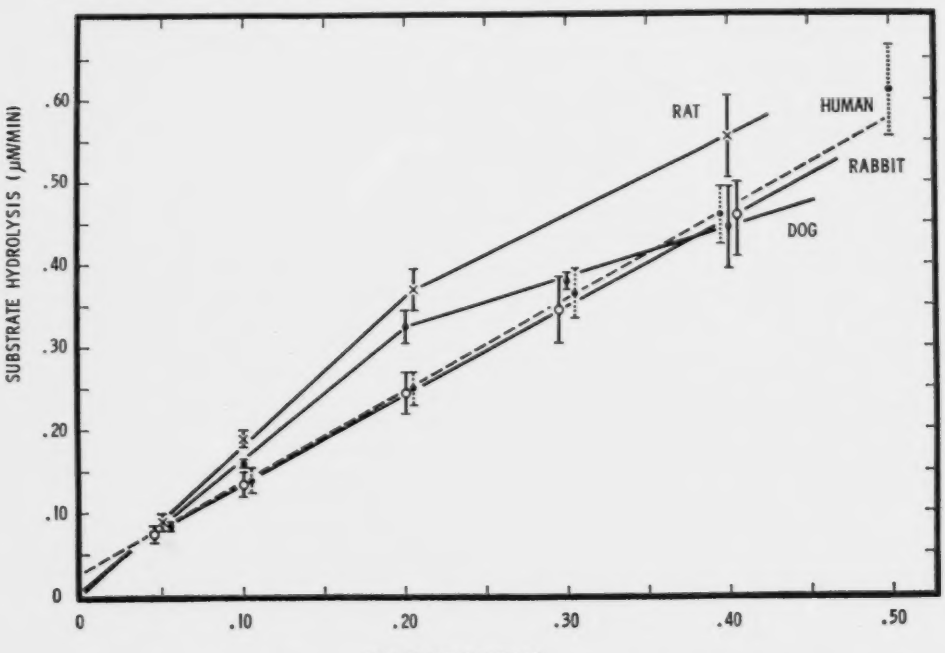

Fig 4.—Erythrocyte acetylcholinesterase activity as a function of enzyme concentration. The conditions of assay were the same as those described for Fig 2.

During the preparation of the hemolysate, the suspension of RBCs and water became a clear ruby-red followed shortly by a gradual development of turbidity with eventual formation of an unidentified massive precipitate. In order to obtain a representative aliquot for analysis, the hemolysate must be mixed thoroughly and a sample taken quickly. A preliminary titration of about three minutes with sodium hydroxide was required for the rat hemolysate; the addition of alkali caused the crystals to dissolve, and at pH 8.00 the mixture in the reaction vessel was completely clarified. The titrator was adjusted so that it stabilized and stopped titrating at pH 8.00, and the substrate was then added for determination of the activity.

Relation of pH to Enzyme Activity.—Studies with human plasma to determine the optimal condition of pH have indicated that the plasma enzyme activity increased steadily as pH increased from a minimum below pH 6.8 to a maximum at about pH 8.2. It has been reported that purified electric eel acetylcholinesterase has maximal activity at about pH 8.2 to 8.4,[16] human serum cholinesterase at pH 8.0 to 8.5,[17] and human RBC cholinesterase at pH 7.5 to 8.0.[15] In this study a pH above 8.0 posed a problem because not only did the activity increase, but the spontaneous hydrolysis of the substrate also increased. The spontaneous hydrolysis started increasing rapidly at pH 8.2, and at pH 9.0 the instrument could not titrate the reaction fast enough. The increased nonenzymatic hydrolysis above pH 8.00 prevented the reaction from being run at the optimum for the enzyme. The assay at pH 8.00 is a compromise to minimize the nonenzymatic hydrolysis while maintaining the enzyme activity near its maximum. It was also necessary to keep the volume of added titrant low in order to avoid extensive dilution of enzyme and substrate in the solution.

Relation of Temperature to Enzyme Activity.—The effect of temperature upon the reaction rate was considered also. By increasing the temperature from 24 C to 37 C, the enzyme activity increased about 50% to 75%. Glick[18] reported an increase of 35% for a rise from 25 C to 35 C. The 37 C temperature was chosen for the assay because this is about the temperature at which the enzyme functions physiologically. The temperature was easily maintained at 37 C with a thermostated reaction vessel and circulating heated water bath.

Comment

The determination as described here fulfills all of the necessary criteria for the determination of cholinesterase. It is rapid, precise, and simple. The conditions of the reaction are easily reproduced, this eliminating much of the variation observed with some other methods. Since the plasma assay can be run within as little as 15 minutes after the blood sample is obtained (or even more quickly if a shorter centrifugation time is used), the determination of reversible inhibition such as that caused by carbamates can be made easily. Previously, it has been difficult to measure accurately the inhibition caused by carbamates because of the rapid reactivation that has occurred in other assays. The determination of RBC cholinesterase required about 45 minutes from the time of sampling because of the time necessary to wash the cells. However, it might be possible to reduce the time required for centrifugation and washing. Another advantage is the recorder presentation of the time course of the reaction which thus allows evaluation of the kinetics of normal and inhibited enzyme to be made easily. The method is particularly useful for the study of the kinetics of the initial stages of the reaction. There is no buffer present, thus allowing convenient study of the effects of pH, salts, and substrate concentration.

Since this study, the Garry and Routh[19] modification of the spectrophotometric method of Ellman and co-workers[20] using acetylthiocholine as substrate has been utilized by us for the determination of rat, rabbit, dog, and human plasma cholinesterases. This method proved to be quite useful since only microliter samples of plasma were needed. Human RBC cholinesterase could be assayed by this method but not that of dogs, rats, or rabbits. The low activity of erythrocyte enzyme in the three latter species necessitated the use of large amounts of hemolysate which interfered with the spectrophotometric readings.

Recently, the Garry and Routh procedure

has been modified by Humiston and Wright[21] and Levine et al[22] for use with the automatic analyzer. These methods, although seemingly accurate and applicable to several species, utilize substrates foreign to the enzyme. These methods are rigidly defined which lessens their usefulness for study of enzyme kinetics.

The titrimetric method is capable of detecting small changes in the plasma or erythrocyte cholinesterase level in laboratory animals. The method, because of its speed, ease of operation, and uncomplicated reaction mixtures, is inherently better suited for application to the rat, rabbit, and dog than are previously available procedures including the manometric method,[4,23,24] the colorimetric method of Fleischer et al,[25] or the potentiometric method,[2] which have at times been used for various animal tissues. The method is flexible and may be varied with regard to enzyme or substrate concentration, pH, or temperature for use with various other tissues not discussed in detail here. For example, the technique has been applied to bird and rat brain by us and to several other tissues by Jensen-Holm et al.[9] There are no theoretical reasons that would prevent its use for determination of cholinesterase in almost all situations.

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PESTICIDES PROGRAM
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A Comparative Study of the Recovery of Metabolized Radiolabelled Pesticides from Animal Tissues

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The continuing development and evaluation of a microanalytical method for the determination of pesticides and their metabolites in human and animal tissues prompted an investigation of the absolute recovery of these compounds. This relationship between the amount of pesticides actually present in animal tissue and the amount extracted by several solvents and solvent systems has now been determined for a variety of tissues. Data will be presented comparing extraction efficiencies of hexane and chloroform:methanol in recovering C^{14}-labelled DDT and dieldrin from the tissues of rats and rabbits dosed with these pesticides. Extraction efficiencies range from 70% for urine specimens to 95% for adipose tissue. The results of this investigation will be related to data obtained for non-radioactive pesticide residues using hexane:acetone and acetonitrile as extraction solvents.

The two pesticides studied represent the extremes of polarity of chlorinated hydrocarbon pesticides generally found in human and animal tissues, namely, DDT and dieldrin and analogous compounds such as DDE and DDD and dieldrin and analogous insecticides such as heptachlor epoxide. Radioactivity measurements on tissue samples were made by combusting the sample, absorbing the evolved $C^{14}O_2$ in basic fluor and utilizing scintillation counting techniques for measuring activity. Quantitative analysis of radioactivity present in the tissue prior to extraction, in the extraction solvent and in the tissue residue remaining after extraction will be discussed. Results obtained with specimens of liver, kidney, brain, adipose tissue, adrenal, gonads, blood and urine will be reported.

El establecimiento y evaluación de un método micro-analítico para la determinación de pesticidas y sus metabolitos en tejido humano y de animales requiere investigación sobre la recuperación absoluta de estos compuestos. La relación entre la cantidad de pesticidas presente en los tejidos animales y la cantidad extraida por distintos solventes ha sido determinada en diferentes tejidos.

Los dos pesticidas referidos en este estudio representan extremos de polaridad de los hidrocarburos clorinados tales como el DDT y compuestos análogos como el DDE, DDD, y dieldrín e insecticidas análogos. Se reportan los resultados obtenidos cuando la investigación se realiza con muestras tomadas del hígado, riñón, cerebro, tejido adiposo, glándulas suprarrenales, sangre y orina.

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Detection of Atypical Cholinesterase by an Automated pH Stat Method

Report of a Family with Both Atypical and Silent Gene Alleles

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Plasma pseudocholinesterase has no known physiological role but is essential for the rapid degradation of succinylcholine, a muscle relaxant used in surgical procedures. Depression of effective enzyme concentrations as a result of hepatocellular disease or exposure to anticholinesterase agents will increase the patient's sensitivity to succinylcholine and prolong postoperative apnea. Synthesis of this enzyme is controlled by an autosomal gene with multiple variant alleles; individuals phenotypically homozygotic for one of these variants will have either qualitatively "atypical" pseudocholinesterase with decreased activity or no enzyme at all, and will thus be more sensitive to succinylcholine. The automated pH stat system described here detects atypical cholinesterase by measuring the degree of inhibition of butyrylcholine hydrolysis by dibucaine. The sensitivity and reproducibility of this technique equals that of standard spectrophotometric methods and its speed is comparable to that of the most rapid screening tests. A family pedigree is also presented, exemplifying a rare genotype in which two siblings are double heterozygotes for "atypical" and "silent" alleles.

Additional Keyphrases: *plasma pseudocholinesterase* • *dibucaine inhibition of butyrylcholine hydrolysis* • *succinylcholine* • *Dibucaine Number*

THE SPECIFIC ACETYLCHOLINESTERASE present at cholinergic junctions of the nervous system is the only enzyme of several known species of cholinesterase to have a clearly defined physiological function. All cholinesterases, however, are inactivated to some degree by anticholinesterase agents, whether as a consequence of medical therapy (for example, with neostigmine) or as a result of overexposure to organophosphate pesticides (such as parathion) or "nerve gas." The inactivation by these agents of acetylcholinesterase in red cells or the nonspecific cholinesterase (pseudocholinesterase) in blood plasma is not responsible for any obvious clinical manifestations. However, this inactivation is of considerable practical importance, for the development of methods for analysis of cholinesterase activity in red cells and plasma (*1*) has provided the physician a valuable laboratory index for assessment of anticholinesterase exposure or intoxication.

Pseudocholinesterase catabolizes several drugs used in anesthesia and in particular is primarily responsible for the very brief effect of the muscle relaxant, succinylcholine. Dependence on pseudocholinesterase for inactivation was known when this drug was introduced to clinical use, and it soon became apparent that the consequence of low enzyme activity was an increased sensitivity to succinylcholine with paralysis and apnea lasting as long as several hours.

Low pseudocholinesterase activity of some of these first cases could be explained on the basis of liver disease, impaired nutrition, or previous exposure to an anticholinesterase agent, but there were others for whom there was no obvious cause. Forbat *et al.* (*2*) were the first to consider the possibility of hereditary disease; they found that a healthy

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young man with succinylcholine sensitivity also had a brother with low enzyme activity.

Genetic studies since then have shown that most individuals with idiopathic pseudocholinesterase deficiency are homozygotic for an autosomal recessive gene that has a gene frequency of 0.019 and a case incidence of about 1 in 2800 in most populations. The primary investigations of Kalow and his coworkers (*3*, *4*) were the foundation for the discovery that this "atypical" gene allele (designated E_1^a) directs the synthesis of an enzyme with low activity but increased resistance to inhibition by compounds such as the local anesthetic, dibucaine. They devised a method of classification based on dibucaine inhibition of benzoylcholine hydrolysis, and by noting the percent inhibition (the "Dibucaine Number") they were able to divide the population into three phenotypes. About 97% of a random group of people tested by this method are in the "usual" category with a normal genotype ($E_1^u E_1^u$) and a Dibucaine Number of 76 to 81. There are about 3% in the "intermediate" group of heterozygotes ($E_1^u E_1^a$) who have a Dibucaine Number within the range of 55 to 69 (this group may display mild increase in succinylcholine sensitivity but ordinarily does not). A very small number of people are in the "atypical" group of homozygotes ($E_1^a E_1^a$) with a Dibucaine Number below 21, low pseudocholinesterase activity, and sensitivity to succinylcholine.

Harris and Whittaker (*5*) reported that sodium fluoride could be substituted as an inhibitor of benzoylcholine [in the method of Kalow and Genest (*3*)] and still permit good separation of phenotypes. They noted during their study, however, that a few cases had "Fluoride Numbers" which were significantly lower than corresponding Dibucaine Numbers, and from this observation they proposed the existence of a third allele(E_1^f). Although this gene is of lower frequency than the "atypical" allele, several patients with moderate succinylcholine sensitivity have been found who were homozygotes or double heterozygotes with other abnormal alleles.

Kindreds have been observed in which there are apparent anomalies in the inheritance pattern. Index cases in these families have phenotypically been "atypical" homozygotes but nevertheless have had one heterozygous and one normal parent, the "normal" parent at times having a borderline or slightly low enzyme activity. These observations could be explained by the postulation of a "silent" allele (E_1^s) which directs the production of a completely ineffective pseudocholinesterase or no enzyme at all; the latter is probably the case.

Liddell *et al.* (*6*) supported this theory by presenting the first reported case of a patient with succinylcholine sensitivity due to the total absence of enzyme activity; several close relatives of the patient had low-normal or mildly depressed levels of pseudocholinesterase activity but normal dibucaine and fluoride numbers. There have since been reports of homozygotes or double heterozygotes for this "silent" allele, but the gene is apparently quite rare.

The method of Kalow and Genest (*3*), which utilizes ultraviolet spectrophotometry to record dibucaine inhibition of benzoylcholine hydrolysis, has been used in almost all major studies of atypical cholinesterase. A certain amount of technical skill and time is required for this technique, however, and efforts have been made to develop tests that would be more rapid, less complicated, and more suitable for large-scale surveys.

Harris and Robson (*7*) have devised screening tests that use filter paper or agar as a supporting phase, α-naphthyl acetate as a substrate, RO 2-0683 (dimethylcarbamate of 2-hydroxy-5-phenyl benzyl trimethylammonium bromide, Roche) as an inhibitor, and a diazo reagent (5-chloro-*o*-toluidine) as a dye indicator. Both "intermediate" and "atypical" phenotypes give a strong reaction and can be distinguished from the "usual" type; heterozygotes and atypical homozygotes may subsequently be separated by determination of Dibucaine Numbers. Morrow and Motulsky (*8*) have adapted this method to spectrophotometric analysis which has the same capabilities and technical requirements as the standard method of Kalow and Genest (*3*).

Swift and La Du (*9*) have reported a simple and rapid screening test which is based on the fact that sodium chloride activates "usual" pseudocholinesterase but not the "atypical" enzyme. Benzoylcholine hydrolysis in the presence of phenol red is assessed by inspection; both "usual" and "intermediate" phenotypes show a positive reaction, whereas those with an "atypical" phenotype or with low enzyme activity for other reasons are negative.

This report describes a method for determination of atypical pseudocholinesterase by using the pH stat procedure and presents a study of a family with both "atypical" and "silent" alleles.

Method

Cholinesterase determinations are performed in this laboratory by an automated pH stat system which is sensitive, rapid, and reproducible (*10*). The mixture for analysis of pseudocholinesterase contains 0.15 ml of heparinized plasma, 0.6 ml of a 0.11 mol/liter solution of acetylcholine perchlorate, and 4.25 ml of physiological saline; it is incubated under nitrogen in a reaction chamber at 37 °C with automatic stirring. The pH of this mixture is maintained at 8.0 by automatic titration with 0.01N sodium hydroxide, this rate of titration being followed by an automatic recorder.

Acetylcholine is customarily used in this method because it serves as a substrate for both red cell

and plasma cholinesterase. Butyrylcholine, however, is a specific substrate for pseudocholinesterase and is hydrolyzed at an even greater rate than acetylcholine. The substitution of butyrylcholine for acetylcholine in the pH stat method requires only minor changes in the method: 0.15 ml plasma is added to 4.6 ml normal saline in the reaction flask. The mixture, under nitrogen, is allowed to equilibrate to 37 °C with automatic stirring. Dibucaine hydrochloride solution (CIBA Pharmaceutical Co.) is added by pipet and the pH then adjusted to 7.5. When the dibucaine solution has been mixed thoroughly, 0.25 ml of a 0.10 mol/liter solution of butyrylcholine iodide is added, and the mixture is titrated at a constant pH of 7.5.

The titration is allowed to proceed until at least 3 min of the recording is linear. The calculation of enzyme activity is made according to the method of Nabb (*10*):

$$\mu\text{mol of choline ester hydrolyzed/min/ml of plasma} = \frac{A \times B \times N \times 1000}{C \times D}$$

where A is the number of recorder units during period of titration; B, ml of titrant delivered by syringe per recorder unit; N, normality of titrant; C, period of titration in min; and D, ml of plasma. The instrument is calibrated so that a recorder deflection of 1.0 represents 0.005 ml of titrant delivered to the reaction flask (Table 1). Control pseudocholinesterase activity is determined in the absence of dibucaine. The Dibucaine Number is the percentage inhibition of the normal activity by dibucaine.

Results

The addition of various concentrations of dibucaine hydrochloride to the reaction mixture (Figure 1) produced inhibition curves that demonstrate distinct separation of the three basic phenotypes. The Dibucaine Numbers for the pH stat method, as shown in Table 2, are those produced by a final dibucaine concentration of 5×10^{-5} mol/liter (0.05 ml of a 10^{-3} mol/liter solution added to the reaction mixture) and are comparable to those of the standard method of Kalow and Genest (*3*). Table 3 gives Dibucaine Numbers and pseudocholinesterase activity for family members of the pedigree shown in Figure 2.

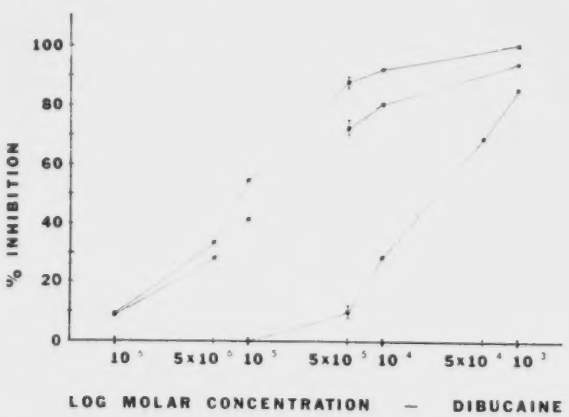

Fig. 1. Inhibition of cholinesterase activity by dibucaine

Reaction of "usual," "intermediate," and "atypical" phenotypes are shown, respectively, by the top, middle, and bottom curves

Table 1. Normal Values for Plasma Pseudocholinesterase Activity[a]

Substrate	Sex	Mean activity ±SD, μmol/min/ml	95% confidence interval
Acetylcholine	M	4.747 ± 1.356	2.035–7.459
	F	3.853 ± 1.140	1.573–6.133
Butyrylcholine	M	7.804 ± 1.647	4.510–11.098
	F	6.487 ± 1.772	2.943–10.031

[a] Sample from laboratory and clerical personnel, 19 men and 16 women.

Table 2. Dibucaine Numbers

Phenotype	Method of Kalow and Staron (4)	pH stat method
Usual	76–81	81–90
Intermediate	55–69	71–77
Atypical	10–21	7–13

Table 3. Pseudocholinesterase Activity and Dibucaine Numbers[a]

Individual	Activity, μmol/min/ml	Dibucaine no.
II_1	2.755	88
II_2	5.762	75
II_3	6.680	86
II_4	4.509	70
III_1	0.718	11
III_2	0.718	9
III_3	8.350	84
III_4	4.760	86
III_5	3.758	73

[a] For family members shown in Figure 2.

Discussion

Our effort to develop a method for detection of atypical cholinesterase was stimulated by our chance introduction to a family with a past history of that disorder. The index case was a young girl who had demonstrated succinylcholine sensitivity several years previously; unpublished studies (Smith, J. C., personal communication) of family members at that time had revealed a pattern suggesting the presence of both atypical and silent gene alleles. We have examined additional members of this kindred during the course of this study, and the

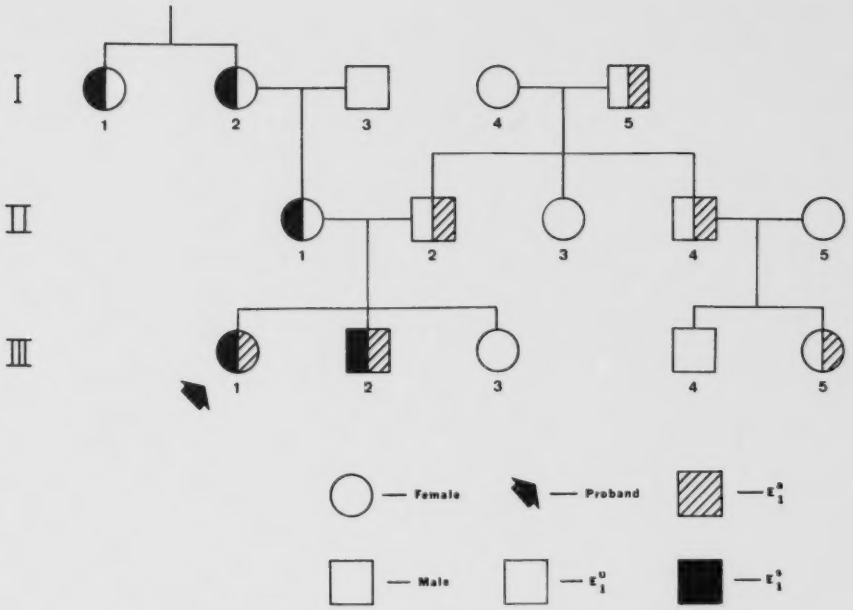

Fig. 2. Pedigree of family with "atypical" and "silent" gene alleles

family pedigree is now included in the report as an addition to the literature of an example of this rare genotype (Figure 2). The silent gene heterozygotes of the first generation were not available for study, but it is understood that their pseudocholinesterase activity was slightly below normal limits. The mother of the proband, who is also presumed to be a silent gene heterozygote, demonstrates pseudocholinesterase activity by the pH stat method that is just below the 95% confidence interval (individual II_1 in Table 3). The brother of the proband, who had never been exposed to succinylcholine, was discovered also to be a double heterozygote during the initial family evaluation; both the children now carry appropriate medical identification.

Although use of the pH stat equipment requires careful maintenance and the skill of trained laboratory personnel, this method has the advantage of precision and reproducibility as well as speed equal to the most rapid qualitative screening test. Any laboratory that maintains and routinely operates this equipment and has the reagents prepared as outlined may analyze a sample of plasma and calculate a Dibucaine Number within 15 min.

This complex of hereditary disorders is uncommon but remains of potential clinical importance to those affected. The availability of a means of detection, such as the method presented here, is of value in the management of patients exposed to succinylcholine. However, it is of greater importance to unsuspecting individuals, discovered by screening tests or family studies, since foreknowledge can obviate the serious potential consequences of prolonged postoperative apnea.

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PUBLICATION

280

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ESTIMATION OF THE ACETYLCHOLINE LEVELS IN BRAIN TISSUE BY GAS CHROMATOGRAPHY OF ACETIC ACID

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INTRODUCTION

The accurate determination of acetylcholine in mammalian brain is of interest to the pharmacologist studying the effects of organo-phosphate insecticides as well as other agents which may alter normal levels of this amine. Bioassay methods have been commonly employed in the examination of acetylcholine concentrations (Ryall et al.). However, bioassay methods are time-consuming and as concluded by Hosein et al. may be subject to experimental variables arising from naturally occurring cholinomimetic materials. Colorimetric methods (Mannering et al; Friesen et al.) have also been used extensively but suffer from extractable interfering compounds and lack of required sensitivity. The accuracy and specificity of the colorimetric and bioassay procedures were improved by the method described by Stavinoha and Ryan which employed the reduction of acetylcholine to ethanol with subsequent identification of the latter by gas chromatography. Their method simplified manipulations and, by comparison to the bioassay procedures, shortened the time required for analysis.

The present paper describes a method which further simplifies the analysis of acetylcholine by circumventing the reduction step and determining directly the acetic acid derived from acetylcholine.

METHODS

Male albino rats (Charles River-CD) weighing 250-300 g were used. Acetylcholine chloride (Merck), acetylcholine iodide (Eastman Organic Chemical), acetylcholine perchlorate (K & K Laboratories), formic acid (Mallinckrodt), acetic acid, ethanol (Chem Service, Inc.) acetylcholinesterase (Hyland Laboratories) and potassium borohydride (Metal Hydrides, Inc.) were used in this study. Water was purified by refluxing a 0.06% aqueous potassium permanganate solution for 24 hours and then distilling the solution in an all glass still.

Extraction and Hydrolysis. The extraction was a modification of the method of Crossland and was similar to that used by Stavinoha and Ryan. Each animal was sacrificed by decapitation, and within 30 seconds the entire brain was dropped into a dry ice-ether bath. The frozen brain was weighed, crushed in a chilled agate mortar, and transferred to a porcelain mortar containing 6 ml of a solution of 0.2% (V/V) formic acid in 95% ethanol. The mortar was cooled with dry ice. The tissue was manually ground for 10 minutes, transferred to 15 ml centrifuge tubes, placed in a refrigerator and allowed to warm to approximately 0°C. Centrifugation was then carried out at 0°C for 10 minutes at 30,000 x g. The sample was decanted and the residue was washed with 5 ml of 75% aqueous ethanol solution containing 0.15% formic acid and centrifuged at 30,000 x g for 10 minutes. The supernatant solutions were combined in a freeze-dry flask and evaporated to dryness under reduced pressure in a 50°C water bath. The residue, taken up in 10 ml of formic acid water (pH 4.6), was centrifuged 30 minutes at 30,000 x g. The supernatant solution, containing acetylcholine, was evaporated to dryness before hydrolysis. Hydrolysis was effected by adding 50 µl of 1 N NaOH and heating for ten minutes at 95°C. The hydrolysate was neutralized with 1 N HCl, brought to 100 µl volume with water, and centrifuged for one minute. The supernatant solution was then ready for gas chromatography.

Gas Chromatography. The quantitative determination of acetic acid and ethanol was carried out with a Perkin-Elmer gas chromatograph equipped with dual hydrogen flame detector and a Sargent SRL recorder. The column was 6' x 1/4" o.d. coiled glass tube packed with Parapak type Q-S 40-50 mesh (Bombaugh, K. J., et al.). The operating conditions were as follows: oven temperatures, 165°C; flow rate of N_2 carrier gas, 45 cc/min. Injections were made with 10 and 50 µl Hamilton syringes. Retention times for ethanol and acetic acid under the above conditions were 2 and 4 minutes respectively. Quantitation was obtained by comparing the peak height produced by the ethanol or acetic acid in the supernatant solutions with the peak height produced by the injection of standards of acetic acid and ethanol.

EXPERIMENTAL

Various hydrolytic procedures were employed to compare results obtained by the method described above with those obtained by other established techniques and to determine that the acetic acid measured is of acetylcholine origin. Brain samples were extracted, the extracts were divided into four aliquots and subjected to the following conditions and hydrolytic procedures:

(1) Alkaline hydrolysis as described under Extraction and Hydrolysis.

(2) Same as (1), but with a known amount of acetylcholine added.

(3) Same as (2), but using the Stavinoha and Ryan procedure for the hydrolysis and reduction of acetylcholine to ethanol with potassium borohydride.

(4) Same as (2), but evaporating the supernatant solution to dryness; subsequently 0.05 ml of pH 8.03 red blood cell buffer (Michel) and 0.01 ml of an aqueous solution containing 0.9 δ pH units (Michel) of acetylcholinesterase were added. After one hour

at 37°C the reaction was stopped by chilling to 3°C. The amount of acetylcholine present in the tissue extract was then determined by gas chromatographic analysis of the acetic acid produced as compared to the acetic acid produced from standard amounts of pure acetylcholine subjected to the same enzymatic hydrolysis. Results of these comparisons are given in Table 1.

RESULTS

The use of the modified alcohol extraction procedure yielded a product free of substances which interfered with the gas chromatographic identification of acetic acid obtained by alkaline hydrolysis of acetylcholine. Values for acetylcholine by acetic acid analysis, although slightly higher, were not significantly different from those obtained by ethanol analysis. The similar results obtained by the different hydrolytic procedures demonstrate that the acetic acid resulting from this extraction procedure is derived from acetylcholine.

Acetylcholine recoveries using the ethanol gas chromatographic method of Stavinoha and Ryan ranged from 93.8% to 98.5% with an average of 97.0%. Recoveries using the acetic acid gas chromatographic procedure described in this paper ranged from 98.0% to 100% with an average of 99.2%. The lower limit of accurate detectability of the acetic acid method is 0.5 µg of acetylcholine/brain. This will vary, however, with the sensitivity of the gas chromatographic detector.

SUMMARY

A gas chromatographic method, which is less complex and requires less time than the method of Stavinoha and Ryan, has been adapted to the determination

TABLE 1. RECOVERY OF ACETYLCHOLINE FROM RAT BRAIN EXTRACT [a.]

Acetylcholine (μg/g Brain)						
By Acetic Acid Analysis (1)	Added	Total	By Acetic Acid Analysis (2)	By Ethanol Analysis (3)	By Acetic Acid Analysis [b.] (4)	% Recovery by Acetic Acid Analysis (2)
4.01	2.50	6.51	6.45	6.32	6.20	99.1
3.97	2.50	6.47	6.49	6.38	6.20	100
3.90	2.50	6.40	6.37	6.11	6.20	99.5
3.85	2.50	6.35	6.26	6.12	6.10	98.6
3.84	2.50	6.34	6.31	6.05	6.10	99.5
3.82	2.50	6.31	6.34	6.28	6.10	100
3.74	2.50	6.24	6.18	6.04	6.00	99.0
3.62	2.50	6.12	6.00	6.03	5.80	99.5
No Tissue	2.50	2.50	2.45	2.41	2.40	98.0
No Tissue	5.00	5.00	4.92	4.81	4.70	98.4

a. Extracts were divided into four equal volumes. One aliquot was analyzed directly, while a known amount of acetylcholine was added to the other three aliquots prior to analysis. The conditions and type of hydrolysis used (1), (2), (3), and (4) is described in the text.

b. Samples were analyzed for acetic acid after hydrolysis by acetylcholinesterase.

of acetylcholine in rat brain tissue. Acetylcholine levels determined by the two methods are similar. Brain tissue was frozen, and then extracted with a formic acid-ethanol solution. The extracts were evaporated to dryness, the residue hydrolyzed with sodium hydroxide and the resulting acetic acid determined by gas chromatography.

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PESTICIDE
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The Identification of Five Unreported Lindane Metabolites Recovered from Rat Urine

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Lindane (γ-hexachlorocyclohexane) has found wide application as an insecticide since the second world war. The metabolic fate of this insecticide in mammals however, is still unresolved. Grover and Sims (1) have shown that rats metabolize both lindane and γ-2,3,4,5,6-pentachlorocyclohex-1-ene, (γ-PCCH), to 2,3,5-and 2,4,5-trichlorophenol which are excreted in the urine as free phenols, sulfates, and glucuronic acid conjugates. The metabolite, 2,4-dichlorophenylmercapturic acid, was also identified in this study (1). Because these metabolites resembled those from the degradation of 1,2,4-trichlorobenzene in rabbits (2) the authors speculated that the metabolism of lindane in rats proceeds via γ-2,3,4,5,6-pentachlorocyclohex-1-ene to 1,2,4-trichlorobenzene. However two isomers of pentachlorocyclohexene have since been obtained from the exposure of houseflies to lindane (3). The isomer whose configuration is uncertain but is referred to as Iso-PCCH, is metabolized more slowly but to a greater extent than γ-PCCH (4). Furthermore, a recent report (5) indicates that rabbits metabolize lindane to o-dichlorobenzene, 1,2,4-trichlorobenzene, the three isomers of tetrachlorobenzene, pentachlorobenzene, 2,3-, 2,4- and 2,5-dichlorophenol, 2,3,5-, 2,4,5- and 2,4,6-trichlorophenol 2,3,4,5- and 2,3,4,6-tetrachlorophenol and pentachlorophenol. These data indicate that the metabolism in mammals is considerably more complex than was previously realized. Through an improved analytical method based on gas liquid chromatography and Coulson electrolytic conductivity detection, it has been possible to characterize five previously unreported metabolites from the urine of rats fed lindane. This paper concerns the isolation and identification of these metabolites.

EXPERIMENTAL

Twenty-four weanling female Sprague-Dawley rats, individually housed in metabolism cages were fed diets containing 400 ppm lindane (obtained from the Perrine Primate Laboratory repository). After the first week

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of treatment, GLC analysis of the benzene extracts of acidified urine samples (pH=2) indicated the presence of at least five unknown metabolites in addition to the 2,3,5- and 2,4,5-trichlorophenols reported by Grover and Sims (1). For the next month, the urine samples were pooled daily, acidified to pH=2 and extracted 3 times with equal volumes of benzene. The combined benzene extracts were then partitioned with a volume of 0.1 N sodium hydroxide equivalent to 20% that of the organic phase. Neutral metabolites remained in the benzene while the polar metabolites were extracted in the aqueous phase. The 0.1 N NaOH was acidified and extracted 3 times with equal volumes of benzene.

The neutral benzene soluble metabolite fraction was concentrated to about 3 ml and chromatographed on an activated silicic acid column under vacuum. The column, previously described in the literature (6), is 36 cm high, 2 cm in diameter and equipped with a 24/40 ground glass joint that fits the collection flasks used in this procedure. The column was packed with 2 cm of anhydrous sodium sulfate at the bottom, 20 cm of silicic acid (activated at 170°C overnight) above this, and another 2 cm of anhydrous sodium sulfate at the top. The column was first washed with 200 ml of hexane. Then the concentrated metabolite fraction was added and the column was developed with an additional 500 ml of hexane. This was followed by 700 ml of benzene, collected in 100 ml fractions. The chlorobenzenes were isolated in the hexane fraction while a compound tentatively identified as a configurational isomer of 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol, (PCCOL), was eluted in the fourth and fifth benzene fractions. This metabolite was further purified by collection of the effluent, at the appropriate retention time, from the gas chromatographic separation of the components of the benzene fraction. The PCCOL was collected in a sufficient quantity and purity to prepare the acetate derivative and obtain unadulterated ultraviolet, infrared, NMR and mass spectra.

Similarly, the polar metabolites were purified by collection of the individual peaks resulting from gas chromatography of the mixture. The polar metabolites were identified as chlorophenols by comparison of the retention times of both the free phenol and the ethyl ether derivative (7) with that of the corresponding standard chlorophenol. In addition mass spectra of these chlorophenols confirmed the identification.

All standard chlorophenols were obtained from Aldrich Chemical Co., Inc. and were purified by recrystallization where necessary.

The gas chromatograph employed was a Tracor MT-220 equipped with a Coulson electrolytic conductivity detector operated in the oxidative mode. The column employed in the analysis and the purification of the chlorophenols consisted of a 6'xl/4" U-tube glass column packed with 5% DEGS on 80/100 mesh Gas Chrom Q isothermally maintained at 180°C. The column used for the collection of the 2,3,4,5,6-pentachloro-2-cyclo-hexen-1-ol contained 5% OV-210 on 100/120 mesh Gas Chrom Q and was isothermally maintained at 140°C. Nitrogen flow was regulated at 90 cc/minute.

Other instruments employed for identification included a model 270 Perkin-Elmer GC mass spectrometer, a Perkin-Elmer 621 and a Perkin-Elmer 257 Infrared spectrophotometer, a Varian HA-100 NMR spectrometer and a Cary-14 recording spectrophotometer.

RESULTS AND DISCUSSION

Rats receiving lindane in their diet were found to excrete metabolites identified as 3,4-dichlorophenol, 2,4,6-trichlorophenol, 2,3,4,5- and 2,3,4,6-tetrachloro-phenol as well as the previously reported 2,3,5- and 2,4,5-trichlorophenols.

TABLE 1

Molecular Masses and Retention Times (Relative to 2,4,5-trichlorophenol) of the Metabolites

Metabolite Identification	m/e (Molecular Ion) (d)	RRT (e)	RRT (f)
2,4,6-TCP (a)	196	0.61	0.40
2,3,5-TCP	-	0.79	0.99
2,4,5-TCP	-	1.00	1.00
2,3,4,6-TTCP (b)	230	1.66	0.98
2,3,4,5-TTCP	230	2.48	2.82
3,4-DCP (c)	162	3.14	1.51 (g)

(a) TCP=trichlorophenol
(b) TTCP=tetrachlorophenol
(c) DCP=dichlorophenol
(d) Molecular mass as determined by low resolution mass spectrometry
(e) Retention times of the free chlorophenols (relative to 2,4,5-TCP) on 5% DEGS at 180°C
(f) Retention times of the chlorophenyl ethyl ethers (relative to 2,4,5-trichlorophenyl ethyl ether) on 5% DEGS at 140°C.
(g) Retention time of the ethyl ether of the unknown identified as 3,4-dichlorophenol (relative to free 2,4,5-TCP) on 5% DEGS at 160°C.

In the identification of these compounds mass spectra of the unknowns indicated that these metabolites were di-, tri- and tetrachlorophenols respectively with appropriate molecular masses (based on ^{35}Cl) (Table 1). Also, in table 1, the retention times of the unknown chlorophenols relative to 2,4,5-trichlorophenol are recorded. These retention times are identical to those of the corresponding standard chlorophenols. The retention times of the ethyl ether derivatives of the unknown relative to the ethyl ether of 2,4,5-trichlorophenol are also recorded. Again the relative retention times of the unknown derivatives are identical to those of the ethyl ethers of the standard chlorophenols.

In addition to the identification of the chlorophenols, a neutral organic-soluble metabolite with the empirical formula $C_6 H_5 Cl_5 O$ and a molecular mass of 267.878 (based on ^{35}Cl) was characterized as PCCOL.

The empirical formula and molecular mass of this unknown was determined by high resolution mass spectrometry. The low resolution mass spectrum (figure 1) had major ion peaks at m/e 268 (M^+); 233 (M-Cl, Base peak); 197 (233-HCl); 169 (197-CO) and 162 (197-Cl).

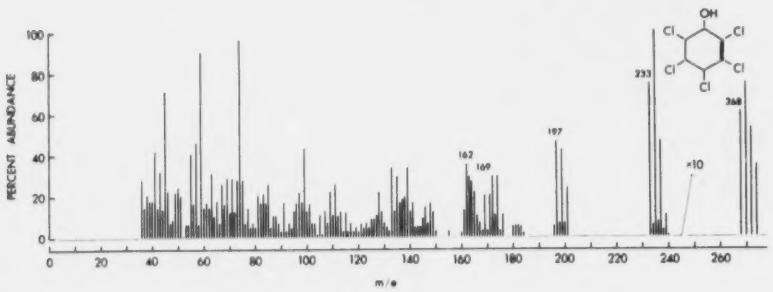
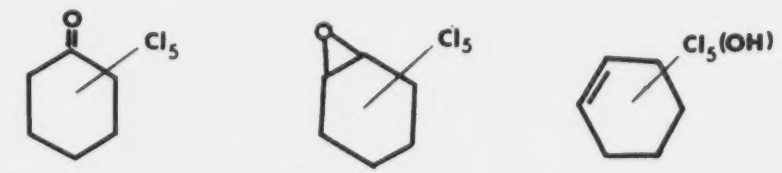


Figure 1. Mass spectrum of PCCOL

Any of the following three structures would accommodate the empirical formula.



I II III

The infrared spectrum of PCCOL, as a KBr pellet, had a broad complex absorption band in the 3400 - 3600 cm^{-1} region (figure 2). That this band indicated the presence of an alcohol was confirmed by a second spectrum of the PCCOL in chloroform (figure 2 insert). In this second spectrum the absorption of the free alcohol at 3600 cm^{-1} is readily apparent. The strong absorption

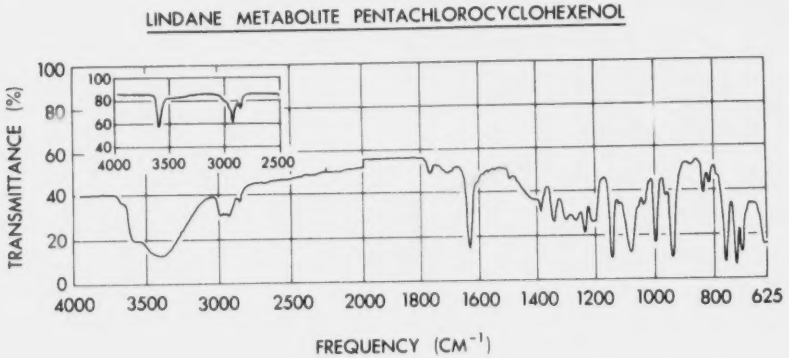


Figure 2. Infrared spectra of PCCOL as a KBr pellet and in $CHCl_3$ (insert)

peak at 1074 cm^{-1} is indicative of a secondary alcohol with $\alpha\beta$-unsaturation (8). The presence of a 1629 cm^{-1} band and the absence of a C-H stretch band above 3000 cm^{-1} suggests a chlorine substituted double bond. There was no stretching vibration which could be attributed to a carbonyl and the 1629 cm^{-1} band could not be accounted for by the epoxide isomer (III).

The NMR spectrum showed a pair of doublets with the first doublet at 473.7 Hz. and the second at 453.9 Hz. The second doublet had an area 3 times greater than the first and each doublet was split by a coupling constant of 4 Hz. These data indicate two chemically inequivalent protons present in a ratio of three to one. This may be interpreted as indicating the occurrence of one proton on each of three halogenated ring carbons and one proton on the carbinol carbon.

Ultraviolet spectrophotometry lent additional support to the suggested occurrence of a double bond with a maximum absorption band at 220 mμ. Furthermore this absorption maximum supports the argument for conjugation between the double bond and the hydroxyl group since cyclohexene absorbs at 208 mμ (9) and dieldrin at 215 mμ. (10). The further shift of the λ max to 220 mμ in the metabolite could be the result of conjugation.

The unknown was reacted with MOX (2% methoxyamine HCl in pyridine) (11,12). This reaction converts keto groups to a methoxime derivative suitable for GLC analysis. That no reaction occurred was indicated by 100% recovery of the parent peak in the GLC analysis. Refluxing the unknown metabolite in ethanol-hydrochloric acid for 4 hours (13) failed to produce a new product based on GLC analysis. This suggested that the compound was not an epoxide. Finally the compound was successfully acetylated (14) and a new product was obtained which had a GLC retention time of 1.68 on 5% OV-210 at 140°C relative to the unknown metabolite. The mass spectrum of the acetylated metabolite gave a parent ion at m/e 310 which would be expected if an alcohol were acetylated. In the infrared spectrum of the derivatized metabolite, the hydroxyl band at 3500 - 3600 cm^{-1} was no longer present while two new bands appeared at 1770 cm^{-1} and 1200 cm^{-1} representing respectively the C=O and C-O stretching frequencies of an ester.

The total amassed data were considered sufficiently conclusive to permit the tentative identification of the neutral metabolite as a configurational isomer of 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol.

Figure 3a illustrates the excretion pattern of the lindane metabolites from a pooled urine sample. The benzene extract of the acidified pooled urine was chromatographed on the 5% DEGS column at 180°C. All the metabolites identified in this paper are readily detected except 3,4-dichlorophenol which is present in trace quantities. The 3,4-dichlorophenol is a minor metabolite found in small concentrations even after extended exposure of rats to lindane. However, after one week of treatment the 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol, 2,4,6-trichlorophenol, 2,3,4,5- and 2,3,4,6-tetrachlorophenol are excreted in greater quantities than either 2,3,5- or 2,4,5-trichlorophenol, the metabolites reported by Grover and Sims in 1965. Figure 3b is the gas chromatogram of the extract used in 3a after partitioning it into sodium hydroxide as described in the experimental section. The polar chlorophenols all reappear in 3b while the less polar PCCOL remains behind in the benzene residue.

The excretion of the metabolites reported here indicate that major revisions are necessary in the currently accepted scheme of lindane metabolism. For example, one would not anticipate the excretion of tetrachlorophenols via the metabolic pathway proposed by Grover and Sims (figure 4). Perhaps the answer may lie in the formation of more than one isomer of the intermediate metabolite γ-PCCH. For example, the second

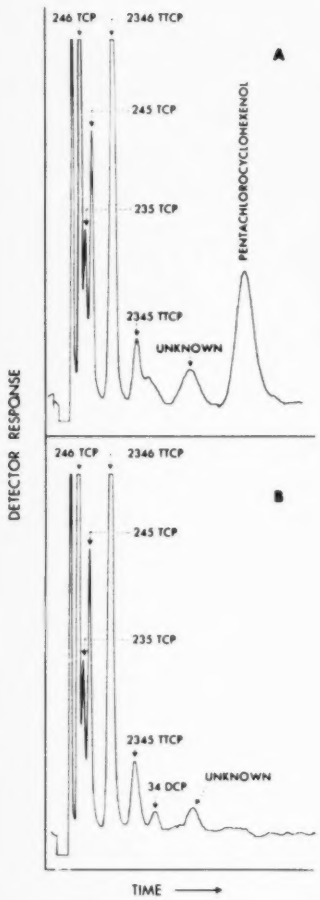


Figure 3. Chromatograms of the benzene extract of a pooled rat urine sample A) Before NaOH partition B) The polar metabolites partitioned into the NaOH.

PCCH (ISO-PCCH) isolated by Reed and Forgash yields 1,2,4- and 1,2,3-trichlorobenzene, 1,2,3,4- and 1,2,4,5-tetrachlorobenzene and pentachlorobenzene upon application to houseflies while the previously reported intermediate, γ-PCCH produced only 1,2,4-trichlorobenzene and 1,2,4,5-tetrachlorobenzene (15). In mammals, the chlorobenzenes are further altered to form the corresponsing chlorophenols. Thus the excretion of

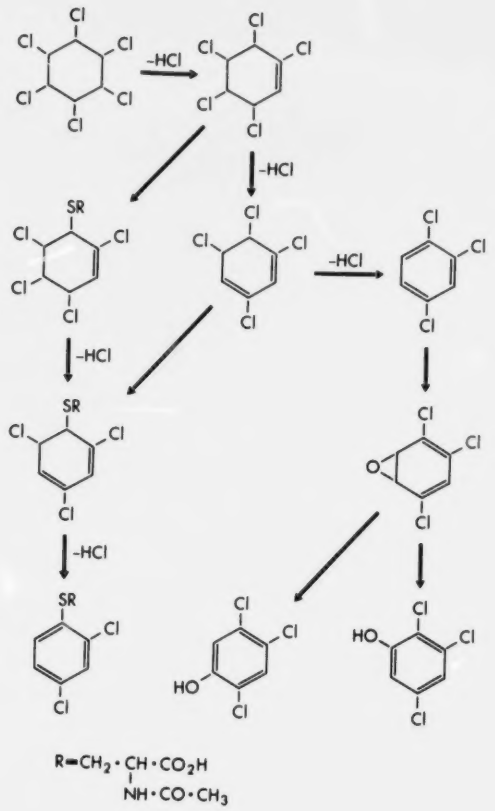


Figure 4. The lindane metabolic pathway proposed by Grover and Sims. Lindane is dehydrochlorinated to γ-PCCH which is further metabolized to either 2,4-dichlorophenyl mercapturic acid or 2,3,5- and 2,4,5-trichlorophenol.

tetrachlorophenols could be accounted for if mammals metabolize lindane via such an intermediate. However, even though γ-PCCH has been identified as a lindane metabolite in flies (16,17,18,19,20,21,22,23,24) plants (25,26), and microorganisms (27) there is no evidence that mammals produce this intermediate. Indeed it would be difficult to account for the formation of the PCCOL with its chlorine substituted double bond, by an initial step involving dehydrochlorination. Perhaps the metabolic mechanism in mammals involves additional pathways or a more efficient pathway than dehydrochlorination to γ-PCCH. Bradbury and Standen state that the first step

in the metabolism of lindane in flies is the removal of one chlorine atom and the formation of a C-S bond (25). Such a route may also occur in mammals, or alternately, the removal of the chlorine atom may be followed by the formation of a C-OH bond. In any case, to be acceptable, a proposed mechanism will have to account for the formation of 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol, an unusual lindane metabolite.

SUMMARY

Previously unreported metabolites from the urine of rats fed lindane have been identified as 3,4-dichlorophenol, 2,4,6-trichlorophenol, 2,3,4,5-tetrachlorophenol, 2,3,4,6-tetrachlorophenol and 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol. While the 3,4-dichlorophenol is a minor metabolite, the others are excreted in greater quantities than either 2,3,5- or 2,4,5-trichlorophenol, previously identified lindane metabolites. The excretion of tetrachlorophenols and PCCOL require a revision in the currently accepted theory regarding the metabolism of lindane by mammals.

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LYMPHOCYTE CHROMOSOME ANALYSIS OF AGRICULTURAL WORKERS DURING EXTENSIVE OCCUPATIONAL EXPOSURE TO PESTICIDES*

PESTICIDE PUBLICATION
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Environmental Protection Agency

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SUMMARY

Lymphocyte cultures from 42 pesticide applicators and 16 controls were scrutinized for chromosomal aberrations during the midwinter ebb in pesticide spraying operations, and again during the peak summer period of intense spraying activity. Cultures prepared from the exposed individuals during the heavy spraying period showed a marked increase in the frequency of chromatid lesions. This trend was especially noticeable among workers exposed primarily to herbicides. A few chromatid exchange figures were also noted among the exposed group. Also discussed is the need for better methods of estimating the potential mutagenic threat, if any, of such observed clastogenicity.

INTRODUCTION

The Idaho Community Study on Pesticides is one of thirteen similar research facilities throughout the United States in areas where pesticides are extensively used. Sponsored by the Environmental Protection Agency, the primary task of this unified investigative effort is to evaluate the effects of pesticide exposure on human health. The Idaho program is now in its sixth year of intense clinical, biochemical and epidemiological scrutiny of a core group of carefully selected volunteers having extreme occupational exposure to various pesticides. An additional parameter currently being examined in detail is the possibility of using cytogenic analysis of lymphocyte chromosomes as an index of pesticide mutagenicity in man.

Cytogenetic analysis of chromosomal aberrations in somatic cell cultures is one of the major methods of testing currently recommended for evaluating chemical mutagenesis in mammalian systems[4,11,14]. Most investigators agree that the dominant lethal test and the host-mediated assay are also important test procedures, and that

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all three should be utilized in confirming mutagenicity in mammals. However, the latter two techniques are not suited for use with human subjects, and somatic cell cultures thus remain the only practical method of assaying morphological evidence of apparent damage to the human genetic material, *per se*. With respect to such cultures, *in vivo* techniques are preferred over *in vitro* tests because they provide the added advantage of taking metabolism into account[8,13]. Although such an approach by itself cannot provide unassailable proof of concurrent gametic involvement, it appears likely that autosomal aberrations may indicate parallel gonosomal events of a similar nature[14]. Even as a purely somatic indicator, analysis of lymphocyte chromosomes would seem highly pertinent when one considers that many spontaneously occurring diseases, certain neoplasms included, are now believed to be caused by environmental pollutants[3].

The tremendous array of agricultural xenobiotics now in common use constitutes a large portion of the various chemical agents to which man is environmentally exposed. The indiscriminate use of such substances would logically present the possibility of a genetic legacy of increased mutation frequency and subsequent human hazard. Increased aberrations of lymphocyte chromosomes have been recently reported[9] in a group of Michigan fruit producers involved in heavy pesticide spraying. Accordingly, we have examined chromosomes of peripheral lymphocytes (leukocytes) cultured from persons selected from our occupationally exposed study group. The prevalence of chromatid lesions among these volunteers has then been compared with that of a control group, both prior to and during the heavy spray season.

MATERIALS AND METHODS

It was initially hoped that sufficient numbers of persons having exclusive occupational exposure to a single chemical series could be obtained. However, because of the great agricultural diversity of this region, such a sampling technique proved to be impossible. Within Canyon County alone, in which most of the exposed participants reside, more than one million pounds of pesticides were applied to crops during 1972 (ref. 10). This figure, irrespective of other agricultural chemicals such as fertilizers, amounts to approx. 4 pounds of pesticides for each acre of farmland. Persons highly exposed to pesticides in this area usually apply a large variety of chemicals throughout the growing season, and multiple exposure is the rule rather than the exception. For these reasons, dose or duration of exposure is another variable that cannot be rigidly defined by such an approach.

Because of their local prevalence in pesticide-related occupations, only male Caucasians were sampled. Persons with recent histories of viral infections, chemotherapy or X-ray treatment were rejected, as were those with histories of malignancies. A total of 16 control and 42 exposed persons, matched as closely as possible as to ages and physical characteristics, were chosen. High exposure was defined as spraying with pesticides for at least 4 h per day on three or more days per week throughout the peak spray season of June 15th through August 30th. The control group was composed of businessman, students, health professionals and teachers who had no history of involvement with pesticides. Ages of the control group ranged from 19 to 64 (mean 39.8). Persons in the exposed group ranged in age from 18 to 70 (mean 38.6) and had from 1 to 25 years (mean 8.5) of prior occupational exposure to pesticides. As shown

TABLE I

PESTICIDES MOST COMMONLY USED BY EXPOSED SUBJECTS IN THIS STUDY[a] (LISTED IN DECREASING ORDER OF FREQUENCY)

Group A Insecticide-exposed		*Group B Herbicide-exposed*
Common name	*Type*[b]	*Common name*
Demeton	OP	2,4-D
Ethyl parathion	OP	Amitrole
Trichlorofon	OP	Atrazine
Naled	OP	Zineb (fungicide)
Malathion	OP	DCPA
Dicofol	OC	Dicamba
Diazinon	OP	Chlorpropham
Endosulfan	OC	Alachlor
DNOC	PH	Metiram (fungicide)
Methyl parathion	OP	Trifluralin
Toxaphene	OC	Maneb (fungicide)
Dimethoate	OP	Picloram
DDT	OC	Diquat
Chlordimeform	OC	2,4,5-T
Oxydemetonmethyl	OP	
TEPP	OP	
Carbaryl	C	

[a] Nomenclature after Berg[2] and Frear[6].
[b] OP, organic phosphorous series; OC, organic chlorine series; PH, phenolic series; C, carbamate series.

in Table I, persons in the exposed group were further divided into two subgroups in accordance with their predominant type of occupational exposure. Group A (mean age, 42.3; mean years of prior exposure, 12.0) consisted of 16 cropdusters, formulators, spray rig operators and farmers who were chiefly exposed to insecticides. The specific chemicals most frequently dispersed by Group A were the organic phosphorous compounds demeton, ethyl parathion, trichlorofon and naled. These and other insecticides applied less frequently by the group are summarized in Table I. Group B (mean age, 36.5; mean years of prior agricultural exposure, 6.3) was comprised of 26 employees of local weed control agencies. These persons were all predominantly exposed to herbicides; especially 2,4-D, amitrole and atrazine (Table I).

Blood samples (10 ml heparinized) were first obtained from all volunteers during the off-season, January–February, 1971, when none of them had been occupationally exposed for a period of at least 30 days. A second blood sample was then obtained from all persons during the period of July through August, which is well into the peak season of pesticide application and represents a common interval of intense daily exposure among the agricultural workers selected. Each person was interviewed extensively prior to both samplings and all pesticide-related information was carefully noted.

Lymphocyte cultures were prepared by means of Difco TC chromosome culture kits (Difco Laboratories, Detroit, Michigan). The technique is based on the procedure previously reported by MOORHEAD *et al.*[12]. Our only modification of their standard procedure was to increase incubation temperature from 37° to 38°, which seemed to facilitate the technique. The samples were cultured for 48 h to obtain cells in the first mitotic division in order to minimize the variability in aberration yield that is some-

times associated with longer culture times[1]. Flame-dried slides were stained with Giemsa and rearranged and coded by a person other than the scorer to avoid bias. 25 metaphase spreads from each person were then examined microscopically for chromosomal aberrations. Only karyotypable cells having widespread chromatids with minimal overlapping were selected for scoring. Photographs were made of all abnormal metaphase figures and karyotyping was performed whenever there appeared to be evidence of obvious chromosomal rearrangement. The criteria of GEBHARDT[7] were followed in the determination of chromatid achromatic lesions (gaps), breaks and chromatid exchange figures.

RESULTS AND DISCUSSION

Other than random loss of chromosomes, heteroploidy was not demonstrated in any of the 1450 mitotic bursts examined. With respect to chromosomal lesions, the majority of achromatic areas (gaps) and breaks were confined to single chromatids. Although isochromatid lesions were occasionally encountered in both control and exposed individuals, this apparent trend toward chromatid aberrations rather than chromosome aberrations would appear to merit further study.

The incidence of chromatid lesions on a per person basis is presented in Table II. The mean number of gaps and breaks per person per 25 cells examined did not appreciably change for the control group at either sampling period. Among the exposed group, however, the mean number of gaps per person nearly doubled in cultures from the midsummer samples, and the mean number of breaks observed per person increased by a factor of 10. The maximum number of gaps found in any single exposed individual (Table II) increased from 2 in the slack season cultures to 5 in the midseason cultures. Maximum breaks for any single exposed volunteer rose from 2 to 6 during the same period. (Different persons were involved in each of the four cases cited.)

TABLE II

SEASONAL VARIATION IN THE OCCURRENCE OF CHROMATID LESIONS

Group	*Number*		*(per person per 25 cells scored)*			
			Chromatid gaps		*Chromatid breaks*	
			Off-season	*Mid-season*	*Off-season*	*Mid-season*
Controls	16	Mean ± S.E.	0.63 ± 0.22	0.69 ± 0.24	0.31 ± 0.12	0.44 ± 0.22
		Range	0–3	0–3	0–1	0–3
Total exposed	42	Mean ± S.E.	0.62 ± 0.11	1.14 ± 0.17	0.17 ± 0.08	1.71 ± 0.24
		Range	0–2	0–5	0–2	0–6
Group A	16	Mean ± S.E.	1.00 ± 0.22	0.75 ± 0.27	0.31 ± 0.18	1.56 ± 0.29
(Insecticide-exposed)	16	Range	0–2	0–3	0–2	0–3
Group B	26	Mean ± S.E.	0.38 ± 0.10	1.38 ± 0.22	0.07 ± 0.05	1.81 ± 0.35
(Herbicide-exposed)		Range	0–1	0–5	0–1	0–6

Those exposed primarily to insecticides (Group A) exhibited a 25% reduction in the mean number of gaps per person in cultures from the high exposure period. However, the mean number of breaks increased 5-fold in these same samples. Members of the group frequently involved with herbicide spraying (Group B) showed an in-

crease of nearly 4 times the mean number of gaps per person during the peak spraying season. Mean breaks per person for Group B increased by a factor of 25 during this period (Table II). Also noteworthy is that the two subjects having the most gaps and breaks, respectively, during the high exposure period were members of the herbicide-exposed group. On the other hand, the markedly low frequency of breaks (0.07 ± 0.05) noted in Group B workers during the slack season invites speculation as to the possibility of induced compensatory protection occurring at this time in the form of enhanced chromosomal repair.

During the July–August scoring of the 42 highly exposed individuals (1050 cells examined), a small number of chromatid exchange figures were noted. These included one Group A quadriradial, two Group C triradials, two Group F quadriradials and a possible Group G triradial. Such interchanges have been found to occur in the general population at a frequency of about one per 3000 cells[5]. The frequency noted in our exposed group approaches one per 200 cells. None of the controls exhibited any of these configurations, but, unfortunately, fewer individuals (400 cells examined) were sampled in the control group. One exposed individual also had a pericentric inversion in the A 2 chromosome. This was confirmed by karyotyping but did not appear in any of the other 25 cells scored for that person.

From these results, it is apparent that the extent of visible chromosomal damage in lymphocyte cultures prepared from the exposed group is considerably greater during the heavy spray season. Without additional correlative studies, however, the possibility of concurrent mutagenic involvement must remain only speculative. Because of the tremendous limiting factors imposed by long generation times, the direct phenotypic surveillance of human populations is restricted to only a few retrospective techniques. One such method would logically include the analysis of existing health statistics. Unfortunately, contemporary vital records are not usually compiled with the geneticist in mind. We have recently attempted to examine the incidence of such parameters as stillbirths, teratisms, retardation etiologies, neoplasms, *etc.*, among agricultural workers in this state, and have had little success because of insufficient data. On a more personal level, another aid in seeking to estimate mutagenicity in suspected populations is the large scale use of detailed interview techniques. However, a major obstacle here is the extent of credibility between researcher and subject.

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Quality Control in the Measurement of Blood Cholinesterase Activities Among Persons Exposed To Pesticides[1]

PESTICIDE
PUBLICATION

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Over the past several years considerable attention has been paid to the health of farm laborers and other personnel exposed to pesticides. Many attempts have been made to use infrequent or periodic blood cholinesterase activity measurements as a means of estimating adverse exposure to anticholinesteratic materials--attempts that, for a variety of reasons often fail to detect any altered status of pesticide workers' health. However, field studies, despite some pitfalls in experimental design, have shown conclusively that farm laborers do suffer depressed levels of cholinesterase activity incurred during the course of their normal employment. Among the most serious considerations in assessing the damage from pesticide exposure is the confidence that can be placed on laboratory cholinesterase activity measurements especially since a wide variety of laboratories with varying degrees of competence in this area are employed in the determinations. Some report values which are empirical; some report activity in terms of unit chemical change with time showing the actual quantities being measured. For all procedures so called "normal limits" are given which presumably apply to non-exposed populations. Acute changes in activity can be detected by any procedure, but more modest alterations can be confirmed only when the sensitivity of the method of assay, the precision and the degree of quality control used in making measurements are assessed fully.

When a single laboratory is involved in cholinesterase determinations results can be compared over a period of time if adequate means of intralaboratory quality control are used. Often, however, it is necessary to compare results on an interlaboratory basis and unless all laboratories involved are under the same regimen of control the measurements between them and over time become questionable, if not suspect. In the absence of interlaboratory quality control "normal limits" are, in essence, applicable only to the laboratory that established them. Since legal claims have arisen on the basis of reported cholinesterase activities that are just beneath the "normal limits" there is no point in using these criteria to define normality when their values are so uniquely

[1] The work upon which this publication is based was performed pursuant to contract No. 70-18 with the Food and Drug Administration.

established. Comparisons of intra- or interlaboratory determinations have been made to illustrate and confirm the need for a quality control program.

Methods and Results

To evidence the necessity of interlaboratory quality control we can first consider some results obtained in a single laboratory, by the same personnel, using reagents from the same source, and employing the same methods but with two different instruments of comparable function and manufacture. Enzyme activities for samples of bovine plasma cholinesterase were determined, with only slight modification in procedure, by the pH-stat technique reported by Nabb and Whitfield . Values determined with one pH-stat differed by more than ± 5 percent from those determined

TABLE 1

Interlaboratory Variation in Cholinesterase Assays of a Series of Human Blood Samples

Sample No.	Cholinesterase Activity (u moles/min/ml)					
	Plasma			RBC		
	Lab 1	Lab 2	Lab 1 / Lab 2	Lab 1	Lab 2	Lab 1 / Lab 2
1	4.24	5.77	0.73	11.9	13.1	0.91
2	2.98	3.88	0.77	11.9	14.6	0.82
3	3.04	4.50	0.68	12.5	12.8	0.98
4	2.90	4.11	0.71	10.5	14.1	0.74
5	4.37	4.81	0.91	13.2	16.4	0.80
6	3.08	4.20	0.73	9.9	14.2	0.70
7	4.31	5.77	0.75	11.7	18.0	0.65
8	4.85	6.52	0.74	10.5	13.8	0.76
9	5.59	8.33	0.67	10.3	12.8	0.80
10	3.24	3.88	0.84	10.7	14.2	0.75
11	4.74	5.84	0.81	13.4	16.5	0.81
12	6.58	8.76	0.75	13.0	14.9	0.87
13	6.25	8.14	0.77	14.4	12.7	1.13
14	1.25	1.67	0.75	11.8	12.6	0.94
15	6.72	8.70	0.77	10.1	11.6	0.87
16	4.41	5.80	0.76	12.2	12.8	0.95
17	2.96	4.19	0.71	12.4	16.5	0.75
18	3.03	4.15	0.73	10.1	11.6	0.87
19	2.76	3.73	0.74	14.0	16.1	0.87
20	4.66	6.21	0.75	11.7	14.6	0.80

with the other instrument in 19 of 31 pairs of measurements (61 percent). There were 8 values (26 percent) differing by more than ± 10 percent and 4 (13 percent) differing by more than ± 15 percent. In another series of 50 pairs of comparable assays on the same two instruments 40 percent differed by more than ± 5 percent, 18 percent of the paired values differed by more than ± 10 percent, while 10 percent differed by more than ± 15 percent. The sensitivity in routine cholinesterase activity determinations on a single instrument should allow no more than 5 percent variation and with some minor differences between instruments perhaps a combined variation of 7 to 9 percent can exist.

If the enzyme assays are performed on an interlaboratory basis and then compared, the problem is further compounded. When two different laboratories each using the pH-stat technique with instruments of the same manufacture assayed aliquots from a series of human blood samples some striking differences appeared (see Table 1). The magnitude of the differences between laboratories in assay values of a given sample, particularly with regard to plasma cholinesterase activity, was sufficient in many cases to evidence adverse exposure if the results on the same sample were, instead, to be considered sequential determinations on the same subject measured in laboratory 2 prior to, and laboratory 1 following exposure. Both laboratories were well experienced in the techniques of assay and both were consistent with a high degree of precision.

To exemplify further the variation in assay values of cholinesterase (bovine serum), this time among several laboratories

TABLE 2

Interlaboratory Variation in Cholinesterase Assays of a Series of Human Serum Samples

Laboratory	Cholinesterase Activity (u moles/min/ml)			
	Sample 1	Sample 2	Sample 3	Sample 4
A	2.7 , 2.7	- -	- -	2.5 , 2.5
B	2.26, 2.27	2.19, 2.24	- -	2.24, 2.24
C	2.5 , 2.5	- -	- -	2.2 , 2.2
D	3.66, 3.52	2.90, 3.03	- -	- -
E	2.83, 2.83	2.57, 2.57	2.70, 2.73	2.40, 2.44
F	3.14, 3.10	2.81, 2.84	3.23, 3.07	2.84, 2.84
G	3.78, 3.78	2.99, 2.84	2.99, 3.14	3.10, 3.10
H	3.40, 3.38	3.26, 3.20	3.41, 3.35	2.90, 2.90
I	2.7 , 2.8	- -	- -	3.0 , 2.8
J	2.02, 2.17	1.88, 1.92	2.01, 2.10	- -
K	- -	2.68, 2.68	2.83, 2.90	2.53, 2.59
L	- -	2.15, 2.20	2.45, 2.55	- -

all using the same method (pH-stat) and exhibiting good precision, we need only examine the data of Table 2.

The difference between maximum and minimum activities measured for anyone of these four samples--47, 42, 41 and 29 percent of the maximum values, respectively--is sufficient to establish cholinesterase inhibition were it not known that the laboratories were assaying portions of the same sample in each of the four sets of measurements. Generally, laboratories were precise and consistent in reporting high or low values for each sample assayed. For any other methods of cholinesterase analysis similar variations are fully expected.

Discussion

Due to the range in analytical results between laboratories even if each used the same methods of analysis, it becomes senseless for each to use the same "normal limits" as a criterion in delineating the absence or degree of adverse exposure to pesticides. Either each laboratory must establish with great care, the sensitivity of measurements by the particular method employed, together with their own realistic normal limits; or alternatively, all laboratories should be guided by a single quality control program. Maintenance of the program must be the responsibility of a central agency and laboratory. As is not uncommon in establishing quality control regulations, this laboratory would supply control samples whose cholinesterase activity is known, at least approximately, but reported as an absolute value to be used by all participating laboratories. In effect, all instruments are then calibrated to the same reference point. Each laboratory would assay the reference sample along with specimens whose values are to be determined and against the stated absolute cholinesterase activity of the reference sample all other values can be calculated. Then, and only then, can meaningful normal limits be set which apply to all laboratories and cholinesterase levels can be compared between the different laboratories, different instruments of similar function and with time.

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A Method for the Determination of 1-Naphthol in Urine

PESTICIDE PUBLICATION

425

Food and Drug Administration
Chamblee, Georgia 30341

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Humans exposed industrially to the insecticide carbaryl (1-naphthyl N-methylcarbamate) excrete relatively large quantities of 1-naphthol conjugated either as the sulfate or glucuronide (1). A colorimetric procedure (2) is generally used to quantitatively determine 1-naphthol in human urine. However, this method lacks both the sensitivity and specificity necessary for determining the relatively small amounts of 1-naphthol excreted in the urine of agricultural workers exposed to low levels of carbaryl. The object of this investigation was to develop a rapid, sensitive method for the determination of 1-naphthol in human urine.

The 1-naphthol resulting from the hydrolysis of carbaryl has been used as an indirect measure of the residue level of the parent insecticide on a variety of agricultural crops (3,4). Argauer (5) described a procedure for chloroacetylating phenols and 1-naphthol for subsequent detection by electron-capture gas chromatography. In a more recent publication (6), Argauer utilized this procedure to determine a number of carbamate insecticides, but indicated that further modification was necessary if the method was to be extended to carbaryl. Since Landowne and Lipsky (7) had previously demonstrated that the monochloroacetate derivative enhanced electron-capture detection to a greater extent than a variety of other haloacetates, it was decided to adapt this derivatization procedure to the determination of 1-naphthol in human urine. To extend the sensitivity of the method, a cleanup step using silica gel adsorption chromatography is included for samples containing less than 1 ppm of 1-naphthol.

Experimental

Apparatus.[1] A gas chromatograph (MicroTek Model 220 equipped with a tritium detector) was operated under the following conditions: 180 cm x 0.4 cm i.d. glass column packed with 1.5% OV-17 and 1.95% QF-1 (w/w) on 100/120 mesh Chromosorb W (dimethyldichlorosilane, high performance); nitrogen carrier gas with a flow rate of 90 ml/min; column temperature 185°C; injection port, detector, and transfer line temperature 210°C.

Centrifuge, International size 2 with metal shield. International Equipment Co., No. 367.

[1] Use of trade names is for identification purposes only, and does not constitute endorsement by the U.S. Department of Health, Education, and Welfare.

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Chromatographic columns, size 22, Kontes No. 420100; concentrator tubes, 25-ml glass-stoppered, ⁵ 19/22, Kontes No. 570050; condensers, ⁵ 19/22, Kontes No. 286810.

Solvents and Reagents. Benzene and hexane, pesticide quality; pyridine, Fisher, redistilled; hydrochloric acid, concentrated, Analytical Reagent; 1-naphthol, Eastman Organic Chemicals.

Chloroacetic anhydride, Eastman Organic Chemicals. Prepare a 2% solution in benzene.

Sodium sulfate, anhydrous. Extract with benzene in a Soxhlet apparatus for 4 hours. Dry and store at 170°C. For use, prepare a 3% aqueous solution.

Silica Gel, Woelm, activity grade I, Waters Associates, Inc. Dry adsorbent for 48 hours at 170°C and store in a desiccator. On the day of use, partially deactivate the silica gel with 1.5% water in the following manner. Add the necessary volume of water to a 125-ml glass-stoppered Erlenmeyer flask, rotating the flask to coat the sides with water. Add the weighed amount of silica gel, stopper the flask, and mix until the water is evenly distributed throughout the adsorbent. Allow to equilibrate for 2 to 3 hours with periodic shaking. Prepare the chromatographic columns just before use.

Use deionized or distilled water, pre-extracted with benzene, throughout the procedure.

Preparation of Standard Curve. From a stock solution of 1-naphthol in benzene, transfer 1-ml aliquots containing from 0 to 500 ng to a series of 25-ml concentrator tubes. Add 5 ml of benzene, 2 ml of the 2% chloroacetic anhydride solution, and 0.2 ml of pyridine. Mix thoroughly and allow the solutions to stand at room temperature for 10 minutes. Wash the solutions 3 successive times with 5 ml of water, centrifuging at 2000 r.p.m. after the last washing. Wrap the concentrator tube with a paper towel before placing in the metal shield for centrifugation. Carefully remove and discard all of the aqueous layer, using a disposable pipet fitted with a rubber bulb. Adjust the volume to 10 ml with benzene and inject 5 µl of each solution (0-250 pg) into the gas chromatograph. Prepare a standard curve by plotting response (peak height) versus concentration.

Procedure for Urine Samples. Pipet 5 ml of urine into a 25-ml concentrator tube, add 1 ml of concentrated hydrochloric acid, and mix well. Fit the concentrator tube with a glass-stoppered condenser and reflux the mixture in a steam bath for 90 minutes, cooling the condenser with circulating ice water. Remove from the bath and cool.

Wash down the sides and tip of the condenser with 2 ml of 0.1 N NaOH followed by 2 ml of benzene. Extract the urine twice with a total of 6 ml of benzene, centrifuging at 2000 rpm after each extraction, and transfer the organic extracts to a clean concentrator tube. Wash the benzene extract with two 3-ml portions of 3% sodium sulfate solution. Centrifuge at 2000 rpm and discard the aqueous layer. Proceed with the derivatization as follows: Add 2 ml of 2% chloroacetic anhydride solution and 0.2 ml of pyridine to the benzene extract. Mix well and let stand 10 minutes at room temperature. Wash the mixture 3 times with 5 ml of water to remove excess derivatization reagents. Centrifuge at 2000 rpm after the last washing and carefully remove all the water. Concentrate the sample to 0.5 ml in a water bath maintained at 40°C, using a gentle stream of nitrogen.

Silica Gel Cleanup Procedure. Add 1 g of partially deactivated silica gel to a chromatographic column which has been lightly plugged with a small piece of glass wool. Prewash the column with 10 ml of hexane and discard the wash. Transfer the concentrated benzene extract to the column with a disposable pipet. Rinse the tube with two 0.5-ml portions of 20% benzene in hexane and add the rinsings to the column. Elute with an additional 8.5 ml of 20% benzene-hexane and discard this eluate. Add 10 ml of 60% benzene in hexane to the column and collect the eluate in a 15-ml centrifuge tube. This fraction contains the 1-naphthyl chloroacetate derivative. Adjust volume to 10 ml with benzene. Inject 5 to 10 µl into the gas chromatograph, determine the peak height, and calculate the concentration from the standard curve.

It is recommended that the elution pattern of 1-naphthyl chloroacetate standard using the silica gel column be verified in each laboratory.

Results and Discussion

A standard of 1-naphthyl chloroacetate was prepared and purified using silica gel column chromatography. A comparison of the electron-capture detector response of 1-naphthol carried through the procedure and an equivalent amount of the purified 1-naphthyl chloroacetate indicates that essentially 100% conversion to the chloroacetate derivative is achieved.

Both infrared and mass spectral data were identical for the purified standard and a compound identified in the analytical procedure as the chloroacetate derivative of 1-naphthol (8). The source of 1-naphthol in this case was from the urine of an individual industrially exposed to carbaryl.

The recovery data summarized in Table 1 were obtained by analyzing human urine samples to which the sodium salt of 1-naphthol had been added. The samples were taken through the entire procedure including the acid hydrolysis and cleanup steps.

TABLE 1

Recovery of 1-Naphthol Added to Human Urine Samples

Amount Added (ppm)	Recovery (%)
0.02	92
0.02	92
0.02	89
0.1	89
0.1	92
0.1	93
0.5	92
0.5	95
0.5	95
1.0	91
1.0	93
1.0	93

Figure 1 illustrates chromatograms of human control urine with and without the addition of 1-naphthol. The peak in A represents 1-naphthyl chloroacetate equivalent to 50 pg of 1-naphthol.

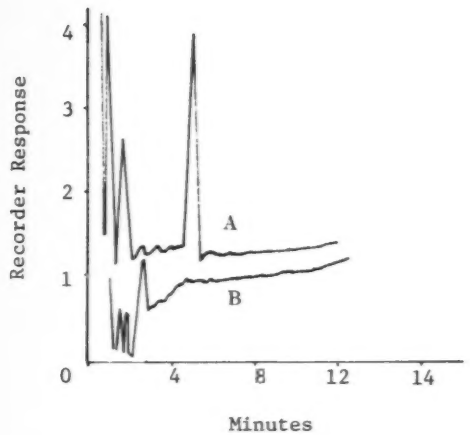

Figure 1. Representative chromatograms. A: Control human urine with 0.02 ppm 1-naphthol added, silica gel cleanup. B: Control human urine, no addition, with silica gel cleanup.

To determine the optimum time for acid hydrolysis, urine samples from the same highly exposed individual were hydrolyzed for times ranging from 30 to 120 minutes. On the basis of the data shown in Table 2, 90 minutes was selected as the length of time necessary to insure complete acid hydrolysis of conjugated forms of 1-naphthol at 100°C.

TABLE 2

Amount of 1-Naphthol Recovered from Urine as a Function of Acid Hydrolysis

Hydrolysis Time (min)	1-Naphthol (ppm)[a]
30	33
60	35
90	48
120	47

[a] Each value is the average of 3 determinations.

Samples of urine, obtained from formulators and farmers exposed to carbaryl at various levels, were analyzed in order to ascertain the applicability of the procedure to a wide range of concentrations of 1-naphthol. Concentrations varied from 6.2 to 78.8 ppm of 1-naphthol in the urine of persons who work as formulators of technical carbaryl at a manufacturing plant, while agricultural workers who used carbaryl for pest control purposes excreted from 0.07 to 1.7 ppm.

Evidence presented in Table 1 and the results of the analysis of urine from individuals exposed at high and low levels indicate that this method can be used to monitor exposure to carbaryl. The limit of detectability has been determined as 0.02 ppm. It is a rapid and sensitive method and the results are reproducible over a wide range of concentrations.

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A Critical Study of the Manometric Assay of Cholinesterase in Rat Blood

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The usefulness of the albino rat as an experimental animal for the study of the toxicity of organic phosphorus insecticides, or other agents which inhibit cholinesterase, is limited by the fact that the level of red cell cholinesterase in the blood of this species is relatively low. With the exception of the titrimetric (Glick, 1937; Scoz and Cattaneo, 1937) or manometric (Ammon, 1933) methods, the usual procedures for the detection of this enzyme in plasma or red cells are not very sensitive. Although the Warburg manometric procedure has been used to assay the cholinesterase of rat erythrocytes (DuBois and Mangun, 1947; Freeman *et al.*, 1949; Pritchard, 1949; Fenwick *et al.*, 1957) or plasma (Beveridge and Lucas, 1941; Mundell, 1944; Myers, 1953; Harrison and Brown, 1951; Ord and Thompson, 1950), a detailed study of the conditions necessary for the assay of these enzymes is not available. It is the purpose of this investigation to provide such an evaluation.

The procedure that is recommended as a result of this study provides an excellent method for the routine estimation of these enzymes in plasma and in red cells. This method can also serve as a reference procedure against which other proposed methods can be tested.

METHODS

Reagents

The inorganic chemicals used in this study were of chemical reagent or analytical grade. Acetylcholine perchlorate (K and K Laboratories[1]), acetyl-β-methylcholine bromide (K and K Laboratories), butyrylcholine perchlorate, heparin (Merck), eserine sulfate (Fisher Chemical), and

[1] K and K Laboratories, 177-10 93rd Avenue, Jamaica 33, New York.

diisopropylfluorophosphonate (DFP) (Aldridge Chemical[2]) were employed in this investigation. Butyrylcholine perchlorate was prepared from butyrylcholine chloride (K and K Laboratories) by a modification of the method described by Bell and Carr (1947).

Acetylcholine (0.036M) for red cells. Dissolve 0.088 g of acetylcholine perchlorate in 10 ml of distilled water. This reagent should be made up fresh each day.

Acetylcholine (0.200M) for plasma. Dissolve 0.490 g of acetylcholine perchlorate in 10 ml of distilled water. This reagent should be made up fresh each day.

Red cell buffer. The red cell buffer is prepared from stock solutions in the following manner. To a 100-ml glass-stoppered graduated cylinder are added 50 ml 3.6% sodium chloride (NaCl), 2 ml 4.6% potassium chloride (KCl), 0.5 ml 8.2% potassium acid phosphate (KH_2PO_4), 1.5 ml 4.8% calcium chloride ($CaCl_2$), 10.5 ml 5.2% sodium bicarbonate ($NaHCO_3$), and 0.5 ml water (per cent based on w/v). Ninety-five per cent nitrogen-5% carbon dioxide is bubbled through the mixture for 5 minutes and then the turbid suspension is stoppered. This mixture is used within 1 hour; if it is necessary to wait for a longer time, the suspension should be regassed. Just before use the red cell buffer is shaken in order to obtain an even suspension. This buffer can be kept overnight at 5°C in a glass-stoppered cylinder. This is a modified Krebs-Ringer bicarbonate buffer.

Plasma buffer. The plasma buffer is prepared in the same way as the red cell buffer except that 0.5 ml of 12.4% magnesium chloride ($MgCl_2$) is used in place of the calcium chloride. The buffer should be regassed if kept more than 1 hour; it can be kept overnight at 5°C in a glass-stoppered cylinder. This is a modified Krebs-Ringer bicarbonate buffer. The stock solutions used in preparing these buffers are stable at 5°C for 2 months.

Isotonic sodium chloride. Dissolve 9.0 g sodium chloride (NaCl) in a total volume of 1 liter of distilled water.

Heparin. Sodium heparin, 1000 USP units per milliliter.

Procedure

For routine assay of the blood cholinesterases 1.5–2.0 ml of heparinized blood are collected from each rat by heart puncture. The sample is

[2] Aldridge Chemical Company, Inc., 2369 N. 29th Street, Milwaukee 10, Wisconsin.

centrifuged for 20 minutes at 2000 rpm in 2.5-ml centrifuge tubes graduated to 0.05 ml. The plasma is drawn off of the red cells and the latter are washed by centrifugation two times with approximately 1 volume of 0.9% saline. During this process the white cells are removed. Finally the cells are diluted to two times their packed volume with 0.9% saline and stored at 5°C in the refrigerator. The plasma is also stored at 5°C; or if one desires to keep the sample for a longer time the plasma can be stored in the frozen state.

The measurement of the cholinesterase activity of the red cells or plasma is carried out in the following manner. To the main compartment of each flask are added 1.6 ml of water and 0.4 ml of plasma or of a 1:1 dilution of packed red cells. The flasks are swirled to mix the contents, and those containing red cells are allowed to stand 5 minutes in order to hemolyze the erythrocytes. Then 0.5 ml of acetylcholine solution (0.036 *M*) for red cells or acetylcholine (0.200 *M*) for plasma is added to the side arm of each appropriate flask. Next, 0.5 ml of red cell or plasma buffer is added to the contents of each appropriate flask. The flasks are gassed for 7 minutes at 37°C with nitrogen-carbon dioxide, 95:5 v v, and then the contents of the side arm are dumped. After 5 minutes' incubation at 37°C the stops are closed and the zero reading is taken. Readings are taken every 5 minutes for 30 minutes.

The rates of carbon dioxide evolution noted with the samples of plasma are corrected for nonenzymatic hydrolysis of acetylcholine by subtracting 0.6 μmole of carbon dioxide from the observed 30-minute rates. The corrected levels of plasma cholinesterase are divided by 2 in order to calculate the standard activity of this tissue, which is expressed as micromoles carbon dioxide per 30 minutes at 37°C per 0.2 ml of plasma. The 30-minute rates of carbon dioxide evolution noted with the red cells are corrected for the blank carbon dioxide released in the absence of acetylcholine by subtracting 0.5 μmole, the average blank, from the observed rate.

The final reaction mixture for the cells contains 0.0166 *M* sodium bicarbonate ($NaHCO_3$), 0.079 *M* sodium chloride (NaCl), 0.00316 *M* potassium chloride (KCl), 0.0017 *M* calcium chloride ($CaCl_2$), and 0.0012 *M* potassium acid phosphate (KH_2PO_4). For the plasma 0.0032 *M* magnesium chloride ($MgCl_2$) is used instead of calcium chloride. A final concentration of 0.006 *M* and 0.033 *M* acetylcholine is used for the red cells and plasma, respectively.

RESULTS AND DISCUSSION

Proportionality between Sample Size and Enzyme Activity

Preliminary experiments showed that the maximal rate of hydrolysis of acetylcholine by the plasma or red cells was obtained with the concentration of the choline ester given in the section on methods. Under

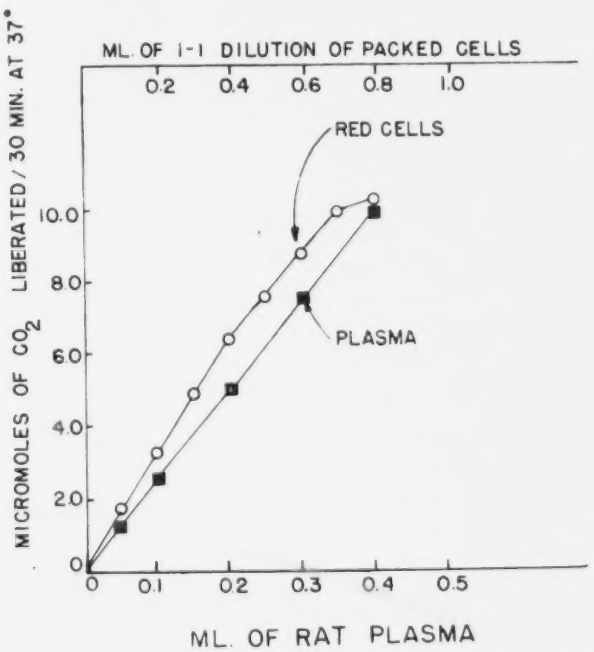

FIG. 1. Proportionality between amount of red cells or plasma and cholinesterase activity in the rat.

these conditions, as is shown in Fig. 1, the cholinesterase activity of the plasma is proportional to the amount of this tissue added within the limits of the amounts of plasma tested, whereas a straight-line relationship between enzyme activity and amount of 1:1 dilution of packed red cells is obtained only for less than 0.5 ml of this suspension. Therefore, the amount of 1:1 dilution of packed red cells used in the assay has been standardized at 0.4 ml. The amount of plasma to be assayed is standard-

ized at 0.4 ml or twice the amount used to define the unit activity of this tissue.

At the concentration of acetylcholine used in this assay, the rate of hydrolysis of this ester which is catalyzed by 0.4 ml of plasma or red cell suspension is linear over a 30-minute period. Therefore, it is possible to read the manometers at less frequent intervals than given in the experimental section and still obtain accurate results. This procedure permits the more convenient assay of large numbers of samples. Duplicate analyses usually check within 1 or 2%.

Stability of Enzymes on Storage

The enzymes in plasma or red cells are stable and can be stored at 5°C for as long as 5 days, and the chilled blood can be kept for a day before centrifugation without loss or redistribution of activity between plasma and red cells. The plasma can be stored in the frozen state without a drop in activity for several months; the erythrocytes should not be frozen since, upon thawing, the cellular protein becomes difficult to dissolve and the suspension cannot be pipetted accurately.

Correction for Carbon Dioxide Evolution from Red Cells

All the results with the red cells given in this paper have been corrected for the evolution of carbon dioxide from these cells in the absence of acetylcholine. This correction ranged from 0.3 to 1.0 μmole of carbon dioxide per 30 minutes from 0.4 ml of 1:1 red cell suspension and averaged 0.5 μmole, or about 10% of the normal level of the red cell enzyme. Freeman *et al.* (1949) had noted this phenomenon in earlier work, but gave no details as to the levels of cholinesterase or the extent of the interference. Attempts to decrease the evolution of carbon dioxide from the cells alone by dialysis against 0.15 *M* saline at 5°C or by aging at 5°C for 5 days were unsuccessful. However, slightly lower values were obtained with a buffer containing calcium instead of magnesium chloride. For this reason, the calcium salt was included in the buffer used for the assay of the red cells.

Experiments in which animals were severely poisoned with parathion so that the *uncorrected* cholinesterase activity of the red cells ranged from 0.3 to 1.0 μmole and averaged 0.4 μmole showed that the blank evolution of carbon dioxide by the cells alone is not inhibited by organophosphorus compounds. If the individual blank for the sample or the average blank of 0.5 μmole of carbon dioxide is *not* subtracted, it should

be realized that cholinesterase activities in the range of 0.3 to 1.0 μmole are of questionable significance.

Normal Levels of Blood Cholinesterases of Rats

The normal levels of red cell cholinesterase of albino rats of the Sherman strain ranging in age from 2 to 8 months are presented in Table 1.

TABLE 1

NORMAL LEVELS OF CHOLINESTERASE IN RED CELLS OR PLASMA OF THE RAT[a]

Age of rats (months)	Parameter	Male rats		Female rats	
		Erythrocyte cholinesterase	Plasma cholinesterase	Erythrocyte cholinesterase	Plasma cholinesterase
2–3	Range	3.2–5.8	1.2–2.7	3.2–6.3	2.9–10.5
	Mean	4.1	1.9	4.2	5.3
	S.D.[b]	0.8	0.5	0.3	2.1
	No. of rats	26	28	22	20
4–5	Range	3.7–4.2	1.6–2.6	3.2–5.1	2.7–16.4
	Mean	3.9	2.1	4.0	7.2
	S.D.	0.18	0.34	0.82	4.6
	No. of rats	6	8	5	7
7–8	Range	4.0–5.1	1.4–2.9	4.0–6.6	6.6–13.7
	Mean	4.5	1.8	5.0	9.3
	S.D.	0.5	0.58	0.92	2.5
	No. of rats	6	6	9	9
Total ages, 2–8	Range	3.2–5.8	1.2–2.9	3.2–6.6	2.7–16.4
	Mean	4.3	1.9	4.4	6.7
	S.D.	0.71	0.49	0.97	3.2
	No. of rats	38	42	36	36

[a] The results are expressed as micromoles of carbon dioxide per 0.2 ml of packed red cells or plasma per 30 minutes at 37° C. All values of the red cell activity are corrected for 0.5 μmole CO_2 evolved from the red cells alone; 0.3 μmole has been subtracted from the plasma levels to correct for the nonenzymatic hydrolysis of acetylcholine.

[b] S.D. = standard deviation.

The value of 2.3 μmole per 30 minutes per 0.4 ml of 1:1 diluted red cells, which was calculated from the data given on 5 rats by Pritchard (1949), is lower than the rates given in Table 1, but is not comparable with values in the present study since Pritchard (1949) used concentrations of red cells and acetylcholine which would produce low activity. As can be seen in Table 1, no difference was noted between the levels of this

enzyme in male or female rats, and the activity of the erythrocyte cholinesterase did not change with age.

In contrast, the plasma cholinesterase of female rats of all ages was found to be much higher than that of male rats, as has been noted by several other workers (Beveridge and Lucas, 1941; Mundell, 1944; Sawyer and Everett, 1947; Harrison and Brown, 1951). Also in confirmation of previous results (Beveridge and Lucas, 1941; Mundell, 1944), it was found that the cholinesterase levels of the plasma of the female rat increased significantly ($P < 0.001$) between the fifth and seventh month of age. The levels of this enzyme found in the present experiment are in excellent agreement with the activities found by other workers (Beveridge and Lucas, 1941; Mundell, 1944; Sawyer and Everett, 1947; Ord and Thompson, 1950; Harrison and Brown, 1951).

Use of Specific Substrates for Assay of Red Cell or Plasma Cholinesterases in Whole Blood of the Rat

It would be an advantage in the present method if centrifugation of the small samples of blood could be avoided and the assay for red cell or plasma cholinesterases could be carried out on the whole blood. Butyrylcholine is a specific substrate for pseudocholinesterase (Cohen *et al.* 1949; Mendel and Myers, 1955), which is considered to be the major cholinesterase present in rat plasma, at least in female rats (Mundell, 1944; Sawyer and Everett, 1947); and acetyl-β-methylcholine is a specific substrate for true cholinesterase (Mendel *et al.*, 1943), the enzyme present in red cells of many species (Mendel *et al.*, 1943). Therefore, experiments were conducted in order to determine the relative rates of hydrolysis of acetylcholine, butyrylcholine, and acetyl-β-methylcholine by separated rat erythrocytes or plasma and thus, whether butyrylcholine and acetyl-β-methylcholine could serve as specific substrates in whole blood for plasma and red cell cholinesterase, respectively. In addition, the effects of eserine, which is an inhibitor (Stedman and Stedman, 1931) of both true and pseudocholinesterases, and DFP, a selective inhibitor of pseudocholinesterase, (Mendel *et al.*, 1943; Hawkins and Mendel, 1947) were investigated. The results of these experiments are presented in Table 2.

It can be seen that butyrylcholine and acetyl-β-methylcholine are not specific substrates for the cholinesterases present in the plasma or red cells, respectively, since both of these choline esters are hydrolyzed by plasma or erythrocytes. The error which would be involved in the

TABLE 2

EFFECTS OF DFP AND ESERINE ON THE HYDROLYSIS OF CHOLINE ESTERS *in Vitro* BY ERYTHROCYTES AND PLASMA FROM MALE OR FEMALE RATS[a]

Tissue	Sex of rat	Choline ester		Rate of hydrolysis as % of acetylcholine rate	% Inhibition with	
		Name	Molarity		Eserine $3 \times 10^{-6} M$	DFP $5 \times 10^{-7} M$
Red cells	Male	Acetylcholine	0.0067	100	100	—
	Male	Acetyl-β-methylcholine	0.0067	40	100	—
	Male	Butyrylcholine	0.033	60	4	—
	Female	Acetylcholine	0.0067	100	100	—
	Female	Acetyl-β-methylcholine	0.0067	44	100	—
	Female	Butyrylcholine	0.033	55	4	—
Plasma	Male	Acetylcholine	0.0067	100	100	43
	Male	Acetyl-β-methylcholine	0.0067	41	100	10
	Male	Butyrylcholine	0.033	75	100	57
	Female	Acetylcholine	0.0067	100	100	50
	Female	Acetyl-β-methylcholine	0.0067	11	100	10
	Female	Butyrylcholine	0.033	96	100	51

[a] The data represent the arithmetric mean of four determinations from pooled rat cells or plasma from five rats 4–4½ months of age. All values have been corrected for the evolution of carbon dioxide from the red cells alone or for the non-enzymatic hydrolysis of the choline ester. In the inhibition experiments, the inhibitors were incubated with the enzyme for 1 hour at 37° C before the substrate was dumped from the side arm to the main compartment of the flask. Concentrations of each choline ester were used that gave the maximal rate of hydrolysis with the enzyme source to be measured with the given choline ester.

use of these esters as specific substrates with whole blood of the rat can be calculated if the assumptions are made that this tissue has a hematocrit of 0.5 and that the sample under consideration has the average activity of these enzymes which are given in Tables 1 and 2. If acetyl-β-methylcholine were used to measure the red cell activity of the whole blood of male or female rats, about 30% of the average value observed would be due to the true cholinesterase present in the plasma. If butyrylcholine were used as a substrate for the plasma cholinesterase in whole blood, the hydrolysis of this ester by the esterase present in the red cells would account for 59 and 26% of the calculated average rate of hydrolysis of butyrylcholine by the whole blood of male and female rats, respectively. Thus, the error involved in the use of butyrylcholine or acetyl-β-methylcholine would be too large to permit accurate measurement of the plasma or red cell cholinesterase in whole blood.

Nature of Cholinesterases in Rat Blood

The data in Table 2 also reveal the nature of the enzymes which catalyze the hydrolysis of the three choline esters. Thus, the hydrolysis of butyrylcholine by rat erythrocytes is brought about by an esterase which is not a cholinesterase, since it is not inhibited by eserine. As might be anticipated from studies of other species (Mendel *et al.*, 1943), the red cells of the rat also contain a true cholinesterase since the erythrocytes showed optimal activity at lower concentrations of acetylcholine or acetyl-β-methylcholine, and the hydrolysis of these two esters was inhibited by eserine. In confirmation of earlier studies which were based on experiments with specific substrates (Mundell, 1944; Mendel *et al.*, 1948; Ord and Thompson, 1950; Myers, 1953), rat plasma was found to contain a true as well as a pseudocholinesterase, since DFP inhibited the hydrolysis of butyrylcholine or acetylcholine, but not that of acetyl-β-methylcholine. The plasma of the female rats contained mostly the pseudocholinesterase, but that of the male animals consisted of a mixture of both types of enzyme in about equal proportions, as had been previously noted in experiments with specific substrates (Mundell, 1944).

The present method employing acetylcholine as the substrate provides an accurate measure of the cholinesterases of the separated red cells or plasma. It does not, however, differentiate between the pseudo- and true cholinesterase present in the plasma of this species. If it is desired to study pseudocholinesterase specifically in the plasma of male rats, butyrylcholine at the concentration given in Table 2 should be used as a substrate with separated plasma.

SUMMARY

A study was made of the conditions necessary for the quantitative assay of the erythrocyte and plasma cholinesterases of the rat by the Warburg manometric method. Butyrylcholine, acetyl-β-methylcholine, and acetylcholine were tested as substrates, but only acetylcholine proved satisfactory. The level of the red cell cholinesterase did not change with age of the rat. The erythrocytes were found to contain an esterase which hydrolyzes butyrylcholine. In confirmation of other workers, it was found that the plasma cholinesterase levels of female rats increase with age and are higher than those of male rats of all ages and that the plasma of male animals contains an approximately equal mixture of true and pseudocholinesterase whereas that of female rats contains mainly pseudocholinesterase.

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A SIMPLIFIED VERSION OF THE MICHEL METHOD FOR PLASMA OR RED CELL CHOLINESTERASE

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SUMMARY

A modification of the Michel method for red cell or plasma cholinesterase has been developed. In this method, the initial pH reading is omitted, and the enzyme reaction is started by adding a mixture of buffer and acetylcholine to the diluted sample. The results are identical to those obtained by the Michel method, but twice as many samples can be analyzed within a given length of time.

The Michel[1] method for the measurement of plasma or red cell cholinesterase has been widely used in studies of men exposed to organophosphorus compounds. This procedure is based on measurement of the change in the pH of a buffer which is brought about by the acetic acid liberated from acetylcholine in the presence of the enzyme under standardized conditions.

The pH of a known amount of buffer and plasma or buffer and red cells is measured (pH_1), and acetylcholine is added to start the reaction. After one hour at 25° the pH of the solution is again measured (pH_2). The difference between the initial and final pH; *i.e.* pH_1-pH_2, is the activity of the sample (ΔpH per 1 h at 25°). It would be a great advantage if this procedure could be simplified. In this laboratory, after the analysis of several thousand samples, it has been concluded that the usual variation in the initial pH of the solution (pH_1) among samples on any given day is well within the error of the method except for an occasional sample obtained at autopsy. In fact, with the exception noted, the change in pH due to the addition of red cells or plasma to the buffer without acetylcholine is negligible. It, therefore, appeared possible to simplify the usual Michel method by elimination of the reading of the initial pH_1 on each of the samples. Accordingly, such a method was devised and, as shown in Table I, the results obtained check very closely to those found with the unmodified Michel[1] method. Twice as many samples can be read on the pH meter in a given length of time with the modified procedure as with the original method

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TABLE I

COMPARISON OF ORIGINAL AND MODIFIED MICHEL METHOD FOR CHOLINESTERASE ACTIVITY OF NORMAL BLOOD

	Plasma cholinesterase		*Red cell cholinesterase*	
	original	*modified*	*original*	*modified*
Number of samples analyzed in duplicate	31	31	20	20
Range of cholinesterase activity (ΔpH/h at 25°)	0.53 – 1.29	0.53 – 1.36	0.55 – 0.83	0.58 – 0.90
Mean cholinesterase activity ± S.D. (ΔpH/h at 25°)	0.94 ± 0.20	0.93 ± 0.19	0.72 ± 0.073	0.75 ± 0.075

since only one reading must be taken per sample. With the modified procedure 25 duplicate samples of plasma and 25 duplicate samples of red cells (100 pH determinations) can be read in an hour. The method has proven useful in thousands of analyses over a period of three years in surveillance of agricultural, industrial, or public health workers who may have been exposed to organophosphates.

EXPERIMENTAL

Red cell acetylcholine–buffer reagent

Accurately mix 1 part of 0.11 *M* acetylcholine perchlorate reagent for red cells (0.68 g per 25 ml final volume of distilled water) with 5 parts of Michel's[1] red cell buffer. The pH should be 8.1.

Plasma acetylcholine–buffer reagent

Accurately mix 1 part of 0.165 *M* acetylcholine perchlorate reagent for plasma (1.02 g per 25 ml final volume of distilled water) with 5 parts of Michel's[1] plasma buffer. The pH should be 8.0.

Both acetylcholine buffer reagents should be used immediately and any unused reagent should be discarded. Acetylcholine perchlorate (K and K Laboratories, Jamaica, New York*) is used instead of acetylcholine chloride as suggested by Michel[1] since the former is not hydroscopic and will keep for at least a year if stored in the dark in a desiccator at 5°.

Other reagents

All other reagents are the same as those described by Michel[1] except that distilled water is used in place of saponin to lyse the heparinized red cells.

PROCEDURE

One to one (v/v) suspensions of red cells are obtained from heparinized blood as described by Michel[1]. If only 1 or 2 ml of blood are available, suspensions of the required accuracy can be prepared by using 2.5 ml glass-stoppered centrifuge tubes calibrated in 0.05 ml divisions. The following procedure is carried out at 25°, and

* Identification of products by supplier is for identification purposes only and does not constitute endorsement by the Public Health Service.

all reagents must be brought to that temperature before use. A well-stirred water bath can be used, but it is more convenient to carry out the assay on a bench free from drafts in an air conditioned laboratory.

Forty μl samples of the 1:1 dilution of red cells are introduced into each of duplicate 5-ml beakers containing 1 ml of distilled water. After the red cells have been allowed to hemolyse for 15 min, 1.20 ml of the red cell–acetylcholine buffer reagent are introduced from an automatic pipette or syringe and the contents of each flask are mixed with an individual stirring rod. After 1 h the pH of the contents of each beaker is read (pH_2, final pH) at the appropriate time interval (usually 30 sec) using a pH meter such as the Beckman Zeromatic which has an electrode with a fast recovery time. The pH meter is rinsed with deionized water and the electrode is gently blotted dry with soft absorbent paper between readings. The initial pH (pH_1) used in calculating the change in pH for each red cell sample is determined by measurement of the pH of a freshly prepared duplicate mixture of 1.00 ml of water and 1.20 ml of the acetylcholine–buffer red cell reagent.

The determination of plasma red cell cholinesterase is carried out in the same manner except that 20 μl of plasma are diluted with 1.00 ml of 0.85% saline, and the initial pH (pH_1) for the plasma is determined by measurement of the pH of freshly prepared duplicate mixtures of 1.00 ml of 0.85% saline and 1.20 ml of plasma acetylcholine–buffer reagent. As a check on the presence of acid or alkaline fumes, the pH of the mixture of water and plasma acetylcholine–buffer reagent is read again at the end of an hour. The pH of this mixture should not change more than 0.08 pH units during this time.

The results are calculated for the red cells or plasma respectively using the appropriate formula $\Delta pH = pH_2 - pH_1$. In agreement with Fryer *et al.*[2], the "a" and "b" corrections of Michel[1] are not applied except at the extreme ends of the range of activities of the two cholinesterases since these corrections are within the range of variation of the method. The readings are not corrected for the change in the pH of the mixture of water and plasma acetylcholine–buffer reagent since the latter correction is included in the "b" corrections of Michel[1].

The 1.00 ml of water or saline and the 1.20 ml of red cell acetylcholine–buffer reagent or 1.20 ml of plasma acetylcholine–buffer must be measured with an accurate dispensing device with rapid delivery. An automatic, all-glass syringe of 3 ml capacity similar to those equipped with Cheney adapters by the Hamilton Company of Whittier, California, but with the stainless steel needle replaced by a polyethylene tube, has been used in this laboratory. However, other types of dispensers, capable of delivering 1.20 ml with a precision of $\pm$ 1%, would be suitable providing that the reagent did not come in contact with metal which might lead to inhibition of the enzymes by traces of metal ion.

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Guidance for Safety Testing of Baculoviruses

U.S. Environmental Protection Agency

The following guidance summarizes the data presently required by the Agency to show the safety of nuclear polyhedrosis and granulosis virus insecticides. (These two groups of insect viruses in the following are referred to as "virus" or "insect virus.")

When testing the safety of insect viruses, primary emphasis must be given to their pathogenicity and not to their toxicity. Pathogenicity is the capability of the microorganism to infect or reproduce, or to cause overt or covert changes, in animals or animal tissue other than target insects. Toxicity means that microorganisms, and/or their by-products, harm the test animal without actual multiplication such as in certain adverse immunological reactions.

Experiments must be designed to support the assumption that the insect pathogen is not a pathogen for humans, other vertebrates, and nontarget invertebrates, nor a hazard to the environment. Proving this hypothesis unequivocally is impossible; however, the required tests cover such a multitude of different approaches that the results will permit an evaluation of the safety of an insect virus and a prediction of the possible hazards.

The safety evaluation studies must be done under the guidance of qualified scientists who, by training and experience, can plan and employ scientifically sound experimental procedures and can assess toxicological, infectious, and pathological manifestations. Any insect virus that causes adverse effects related to overt or covert infection or reproduction in nontarget test animals should not be considered as a potential control agent. Nothing in this guidance shall be construed as relieving the investigator of these responsibilities.

The requirements for safety testing depend on the proposed uses of the virus. The impact of the virus on the environment and other organisms must be considered. In specified instances and with limited use, a temporary exemption from the requirement of a tolerance is granted in accordance with an experimental use permit for experimental field trials of the virus. Generally, and when used on an experimental basis on food crops, the acute and subacute feeding studies must be completed, as well as eye and dermal irritation/toxicity tests, with the virus and the formulation. Depending on the type of use and application, inhalation studies also must be completed. The insect host spectrum of the virus must be studied. The possible effects on fish, birds, and invertebrates (other than insects) must be determined. If the registrant feels that any of these requirements should be waived for uses on nonfood crops (and possibly food crops), an argument for waiver must be presented.

The virus preparation used for safety testing must be the same technical product as that which is subsequently produced and used commercially. The virus must not be inactivated by components of the diet with which it is fed to test animals. Certain safety tests, such as eye and skin irritation tests and acute exposure feeding tests, may also be required for the formulation, especially in the case where materials not previously tested and approved are used in the formulation.

The virus must be an insect pathogen which is identified taxonomically according to host spectrum and serological, biochemical, and biophysical tests. The fungal and bacterial contamination of the technical virus due to production procedures must not exceed the limits which can be maintained under sanitary manufacturing conditions. More importantly, human and mammalian pathogens (e.g., *Salmonella*, *Shigella*, and *Vibrio*) and microorganisms producing toxins known to affect mammals must be absent from the technical products.

Quantitation of virus must be based on bioassays. Standardization of bioassays is difficult; the variability of the assay, however, should be known.

Since the following guidance is subject to revision according to the most recent knowledge about viral insecticides, it is recommended that petitioners confer with Agency officials before starting the tests described below.

Protocols should be designed so that the data can be evaluated statistically. The actual number of test animals to be used is generally not specified in order to permit the most individualized approach to each test. Where numbers are indicated, they are meant as suggested minimal numbers.

SAFETY TESTS

A. Dosage

Since the concepts of no-effect level and LD_{50} for species other than the natural hosts cannot be applied to insect viruses, the following procedure must be used to determine oral dosages. The dosage calculated below is arbitrary; it will, however, introduce a liberal margin of safety since an

individual will very likely never be exposed to the quantity of active virus applied to 100 acres. Introduction of lower dosage levels will not provide additional knowledge about the possible pathogenicity of the virus.

The quantity of biologically active virus expected to be applied to 100 acres is determined. In designing the laboratory animal experiments, it is assumed that a 70-kg man would be exposed to this quantity, and the presumed 100-acre equivalent exposure of a man is calculated in active virus per kilogram of body weight. The experimental animals are then exposed to the same dose adjusted to their weights. In acute tests, the entire amount is given as a single dose; in 90-day feeding studies, the total quantity is fed in 90 portions. If the determined amount of virus is excessive, especially for a single dosage, a 10-acre equivalent can be used. For a single dose, the amount of virus should not exceed 50% of the daily food intake of the experimental animal; for multidose experiments, the addition of virus to the diet should not interfere with the nutritional needs of the test animals.

The above dosage is meant for feeding studies and with technically necessary adjustment for dermal exposure studies. For dosages used in other tests, such as inhalation and tissue culture experiments, one should refer to the respective sections below.

B. Single-dose exposures

1. Feeding studies

a. Schedule of feeding and sacrifice. The insect virus is administered in a single dose to young adult laboratory animals of both sexes (two species). Some animals (at least one of each sex) should be sacrificed shortly after feeding of the virus, and others should be sacrificed later, at intervals of hours to several days, until the termination of the experiment. Shorter intervals should be used at the beginning of the test. Some animals (at least three of each sex) should be left to survive for at least 35 days to make serological tests meaningful. Animals (one mammalian species) which are on an effective regimen of immunosuppressive drugs must be included in this study (one control group and one group which is exposed to virus).

b. Data to be collected from experimental animals.

i. Growth, food consumption, general appearance, mortality, and morbidity.

ii. Attempts to isolate and detect insect virus inclusion bodies or virions in gastrointestinal tract, blood, urine, and selected body tissues which would normally be affected by a viral infection (e.g., lymph nodes, kidney, cerebrospinal system, spleen, liver). Use the most specific and sensitive assay for virus detection.

iii. Body temperature, measured twice daily at the same times each day.

iv. Antibody response to viral proteins.

v. Hematology, urinalysis, organ function tests, organ weights, and gross and microscopic pathology. It will suffice to investigate these items at the end of the experiment and possibly at the midterm.

vi. All animals dying before termination should be examined grossly and microscopically, and the cause of death should be determined, especially if a microbial etiology is suspected.

2. Eye irritation study. Usually performed on six albino rabbits; observation after 1, 2, and 3 days, and longer if irritation persists.

3. Dermal toxicity and skin irritation. Single application of insect virus to intact and abraded skin (rabbit) with observation for 21 days; necroscopy of animals after 21 days, with gross pathology and histological examination in cases where pathological changes of skin or organs are noted.

4. Inhalation study. Administration of virus product as dust or aerosol, by single exposure. The concentration of the aerosol and the duration of exposure must be such as to reflect an unusually high exposure level of a man using the insecticide spray. Shortly after exposure and at intervals thereafter, the lung and other tissues of the respiratory tract must be assayed for the presence of virus. The residual virus must be isolated from

the respiratory tract by bioassaying, with a sensitive insect larval test, tissue, and aspirated pneumobronchial washings. Some animals must be observed for at least 2 weeks, after which time a terminal necroscopy with gross pathology and histological examination of the respiratory system must be performed.

5. **Human, primate, and nonprimate tissue culture studies.** Tissue culture studies, though not representative of the conditions of the intact animal body, must be used to explore the safety of insect viruses, i.e., the absence of viral multiplication, inapparent association with the cell, or other interference at the cellular level. Primary or other diploid cells of human, primate, and nonprimate origin should be exposed to purified, infectious virus (free from insect fragments and bacterial contamination).

The optimal conditions, such as multiplicity of infection, buffer system, optimal temperature for adsorption and penetration, for infecting tissue cultures must be determined using host insect cells. The homologous tissue culture system must show a successful infection and will serve as positive control. If possible, the infectious unit(s) should be isolated and identified. In cases where homologous insect tissue cultures are not yet available for testing the infectivity of the inoculum, the vertebrate cells should be challenged with infectious hemolymph, because it presumably contains infective units capable of infecting tissue culture cells.

Some duplicate cultures of the exposed vertebrate cells also must be maintained at lower-range temperatures (about 30 C, or lower if possible) to assess the possible interactions of insect virus under conditions which approach the optimum for its invasion, multiplication, and interactions in living insects or insect tissue cultures.

Techniques of transfection (forced entry and interaction of viruses and virus genomes with nonpermissive cells) also can be applied; for more detail, refer to the section on long-term studies.

Observations. For cytopathic effects and the formation of inclusion bodies in the living and fixed and stained cells, but also for more subtle changes such as cell transformation and interference with viruses which would normally grow in the tissue cultures.

Three blind passages must be performed in each cell type, and each passage should be maintained as long as possible, but at least 14 to 21 days. At each passage level, a chromosome analysis of the exposed and control tissue culture cells must be performed. The last passage also must be tested by quantitative bioassay in susceptible insect larvae to determine whether multiplication of infectious insect virus (inclusion bodies or virions) occurred in the tissue cultures.

The fluorescent-antibody technique, for example, or other sensitive methods must be applied to a selected number of exposed tissue cultures, to determine whether viral antigens or subunits, which would not be detected by bioassays, were produced.

C. Multiple-dose exposure.

1. **Oral exposure by feeding or by direct dosing.**

   a. **Number of species.** At least two, one a nonrodent.

   b. **Duration.** Ninety days.

   c. **Observations.** Growth, food consumption, general appearance, mortality, morbidity, hematology, urinalysis, organ function tests, organ weights, gross and microscopic pathology. Tissues examined should be those described in the WHO Technical Report Series No. 426 (1969), with special emphasis on those expected to be affected by viral infections (intestine, nervous tissue, lymphatic tissue, spleen, liver, kidney). All animals dying before termination should be examined grossly and microscopically as well as for microbial organisms which might be the etiological agents of death. The antibody (circulating) response of a sufficient number of animals should be studied after 30, 60, and 90 days.

   Several virus diseases of vertebrates are known where the fetus appears to be the most affected target. These diseases also often show no or minimal clinical signs in the adult. The search for a teratogenic effect after viral

exposure per os, during the gestation period, therefore, could be a very sensitive method for the detection of an adverse effect on the adult (female).

2. **Skin sensitization.** Multiple intradermal injections of virus material and/or patch application. For allergenicity tests, guinea pigs should be used preferentially as test animals. Dosing and observation for 3 weeks with challenge injections 3 to 7 days later to assess sensitization. In case of extreme sensitization, the antibody response of the animals should be investigated.

3. **Respiratory sensitization and inhalation toxicity.** Guinea pigs must be exposed to dust or aerosols of the insect virus for 1 h each, four or five times per week for 3 weeks. One week after the last exposure, the animals are challenged by the respiratory and dermal route to assess allergic reactions. At termination of the experiments, the animals are also investigated for pathological changes of the pneumobronchial system.

D. Long-term studies.[1] The present knowledge concerning the interaction of insect virus with permissive and nonpermissive cells does not allow a final conclusion on whether or not an interaction at the molecular or genetic level is possible. Such interactions would most likely manifest themselves in long-term studies and not in acute and subacute ones. It is therefore proposed that a two-generation lifetime oral exposure study be conducted to assess possible effects on animals exposed to insect viruses from the time of their conception until the end of their life span. The study will be carried out according to standard protocols for long-term carcinogenicity studies.

The basic question, can or will insect viruses interact with vertebrate cells, still remains unanswered. If it could be answered in the negative, carcinogenesis, mutagenesis, and reproduction studies could be omitted. The following approach to resolve the question is suggested. (The important aspect of the approach is that specific and sensitive detection methods for molecular or genetic interactions are used. Such methods have not been developed or applied to insect virus systems.)

1. The invertebrate cell-virus system must be defined as a control. The uptake and behavior, such as uncoating, and the growth curve and maturation of the virus and virus components must be studied. The infectious unit or units must be defined.
2. Human, vertebrate, and invertebrate cells should be challenged using the conditions found most suitable for the permissive control system outlined under 1.
3. Artificial methods should be used to get infectious units into nonsusceptible cells, such as DEAE-dextran, solid carriers (kaolin, bentonite), and osmotic or nutritional manipulation. After both the simple and artificial challenge of cells, observations must be made for overt infection, persistent infection, or inapparent infection. In order to identify an inapparent infection, an attempt should be made to detect cell surface changes (antigens) and intracellular soluble antigens, for example. Viral interference tests and observation for morphological changes also would indicate the presence or absence of inapparent infections. DNA hybridization may be another suggested but untried method to detect subinfectious interaction.

E. Beneficial insect, wildlife, and plant toxicity. The following toxicity tests are designed to assess the possible adverse impact on nontarget invertebrate and vertebrate species and plants exposed to virus in the environment. The dose must reflect the level of insect virus to which the environment is exposed under the expected conditions of virus insecticide use (for example, infectious virus units per liter of water or per square meter of forage area). A level 100 times this and one intermediate level must also be tested. Some acute studies customarily required for the assessment of fish and wildlife toxicity seem of little value in the case of insect viruses; subacute studies will give more assuring results.

1. **Effects on invertebrates.** The host spectrum of the insect virus must be deter-

[1] The purpose of long-term studies is to help in the prediction of possible effects on man or the environment resulting from prolonged (life-long) low-level exposure, such as intake via food. Since insect viruses are a natural component of the environment, it is impractical to be concerned about exposure to natural levels of virus. Quantitative determinations of virus levels (see section F, subsection 2) thus may be sufficient to determine whether or not a possible long-term hazard from the use of insect viruses exists, especially in those cases where the use of viruses does not significantly increase, or even decreases, the level of exposure.

mined (see identification of pathogen). Honeybees and other beneficial or aesthetically and commercially important invertebrates, as well as parasites and predators of the target insect, should be included in the experiments which determine the host range. Data for evaluating the effects on nontarget insects can be obtained through laboratory experiments as well as field sampling procedures.

2. **Effects on fish (and other aquatic vertebrates).** Representative fish species must be exposed to insect virus. The virus must be added in suspension to the water and in the form of diseased larvae to be eaten by insectivorous fish. The period of exposure and observation must be extended to 30 days, after which time an autopsy of the fish should be performed.

   An alternative or extension of the fish exposure studies would consist of exposing fish and amphibian tissue culture to virus. The problems of detecting virus interaction at the cellular level are pointed out in the section on mammalian tissue culture studies and long-term studies. These same problems apply to fish tissue cultures and must be carefully considered by the investigator.

3. **Effects on birds.** Two species of birds, including at least one insectivorous species, must be exposed to virus by the oral route. Since birds may act as a vector of insect viruses via their droppings, the fate of the virus in the birds' digestive tracts must be studied. Birds, in fact, may be included in the acute studies listed under B.1 or C.1 since some species are expected to eat large quantities of virus when they feed on diseased insects.

   Bird reproduction studies are not required; the Agency, however, retains the option to require such studies in case a review of data indicates that a problem may exist.

4. **Effects on plants.** No controlled studies to investigate effects on plants are needed. During experimental use, however, protocols must be designed to detect phytotoxic effects, should they exist. The finding of these observations must be reported.

F. **Monitoring.** Monitoring of effects and impacts of pesticides is an important aspect of their overall safety. Logically, monitoring cannot be a prerequisite for experimental use; rather, it must be undertaken during experimental use and extended to periods of full use. Continued monitoring of the environment and exposed humans will provide additional assurance that a virus which has undergone safety tests elicits no long-term adverse effects which might have escaped observation. Effective monitoring depends on the development of sensitive and specific assay techniques which presently are not completely finalized. The following, therefore, is a suggested and possibly crude approach which should be changed once the necessary experience has been gained.

1. **Human monitoring.** Human exposure experiments are not required. However, a research or industrial team working with potential virus insecticides provides an excellent opportunity to check the responses (especially serological and allergic responses) of exposed humans. Surveys and long-term follow-up studies on such occupationally exposed persons are, therefore, highly recommended. Presently, only general health surveys including neurological function tests can be suggested. In cases where specific serological tests are available, sera of exposed humans must be assayed; where tests are not yet available, sera should be stored frozen for later use. It is important that a series of serum samples of each individual be available.

   Prospective and retrospective epidemiological studies of high-exposure groups may also aid in the assessment of possible ill effects on humans.

2. **Environmental monitoring.** The most important aspect of environmental monitoring is an investigation of virus levels in nature (host plants of target insects, water, etc.) and the development of techniques and methods to perform this type of monitoring activity. A variety of conditions may exist and have been documented; for example, epizootics in forest insects produce hundreds of times the amount of virus usually applied to forests, and virus levels on food crops may be less after purposeful introduction of the virus than after natural epizootics late in the growing season. The assessment and monitoring of "natural" virus levels and virus levels after use in relation to the amount applied are very important aspects of the safety evalua-

tion. Observation for unusual ecological occurrences in treatment areas may be another empirical approach to environmental monitoring. Systematic assessment of wildlife species, including blood collection of hunted species for serological tests, should also be undertaken.

G. **Changes and/or additions of experimental designs.** The proposed safety testing experiments for insect viruses are still in need of improvement. Quantitative virus isolation, serological tests, biochemical-biophysical virus identification, and specific detection methods at the cellular level are some of the areas where progress is needed. Therefore, it is the duty of every scientist as well as Agency official involved in this problem to contribute to the improvement of safety testing, and suggestions based on scientific evidence are needed. Changes resulting from new scientific data should be submitted to the Agency and discussed with Agency officials to assure that any new approach satisfies the Agency's requirements.

Administration of virus to animals via injection is not strictly required, since such procedures have little semblance to natural exposure patterns except in cases where biting insects are to be controlled. Tests using parenteral administration of virus, however, are highly recommended, and they could be combined with the needed antiserum production. The criteria for monitoring the animals and the fate of the virus should closely follow the criteria listed under acute feeding studies.

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Comparative Stimulation of γHCH Metabolism by Pretreatment of Rats with γHCH, DDT, and DDT + γHCH[1]

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Comparative Stimulation of γHCH Metabolism by Pretreatment of Rats with γHCH, DDT, and DDT + γHCH. CHADWICK, R. W., CRANMER, M. F., and PEOPLES, A. J. (1971). *Toxicol. Appl. Pharmacol.* **18**, 685–695. Pretreatment of rats with γHCH, DDT, and DDT + γHCH accelerates the metabolism of γHCH. A single oral dose of ^{14}C-γHCH was administered to all animals after 2 wk of pretreatment. The treated rats excreted significantly more radioactivity and stored significantly less ^{14}C-γHCH than the control animals. In addition to significantly larger livers, the treated rats exhibited higher cytochrome P-450 content and greater in vitro enzyme activity than the control animals. The self-induction of γHCH metabolism, which has not previously been reported, could be of major importance in establishing a steady-state relationship between exposure and storage of such pesticides by mammals. Both quantitative and qualitative differences in γHCH metabolism are observed between the treated groups of rats. DDT pretreatment results in a 35% higher excretion of radioactivity than γHCH pretreatment. From the distribution of radioactivity it is concluded that DDT pretreatment produces proportionately more neutral and weakly polar but less free acid-type γHCH metabolites than γHCH pretreatment. Microsomal mixed function oxidases appear to be primarily responsible for the 3- to 4-fold increase in γHCH metabolism by the DDT-pretreated rats. However, the γHCH-pretreated rats exhibit significantly higher glucuronyl transferase activity, an enhanced GSH-dependent degradation of γHCH, and a lower level of the mixed function oxidases than DDT-pretreated rats.

Results of this study suggest that the mechanism by which γHCH induces its own degradation differs from the mechanism through which DDT stimulates γHCH metabolism.

Extensive contamination of the environment, concentration by food chains, and storage in animal tissues have generated increasing public concern over the long-term effects of pesticidal compounds to man and animals. Pesticides generally are encountered in the environment as mixtures of compounds, and the interaction of multiple contaminants with biological systems complicates the prediction of a response to any single toxicant. When pesticide combinations are administered to experimental animals, there is evidence that the storage (Street, 1964; Cummings *et al.*, 1966, 1967; Street and Blau,

[1] Presented at the Society of Toxicology Meeting, Atlanta, Georgia, March 1970.

1966: Deichman *et al.*, 1969), excretion (Williams *et al.*, 1964; Langlois *et al.*, 1964), and metabolism (Street and Chadwick, 1967; Gillett *et al.*, 1966; Triolo *et al.*, 1966) of these compounds are altered. Since these responses are indicative of animal ability to metabolize both foreign and endogenous compounds, any modifications should be carefully investigated.

This study was conducted to investigate possible alterations in the detoxifying mechanisms of mammals exposed to two widely encountered insecticides, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), and γ-hexachlorocyclohexane (γHCH).

METHODS

Twenty-four weanling female Fisher 344 rats were randomly assigned to one of 4 treatments in a 2 × 2 factorial experiment involving DDT and γHCH. All groups received their respective treatments by daily oral injections of peanut oil solutions. The treatment groups received either 2 mg of DDT, 2 mg of γHCH, or 2 mg each of both DDT and γHCH; the control group received only the peanut oil. On day 14 of the experiment, all animals received 2 mg of γHCH (containing 2.0 μCi of ^{14}C-labeled γHCH[2]) in place of the previous treatment. The feed in this experiment consisted of a purified vitamin A test diet.[3] A vitamin supplement[4] was also administered in the drinking water. All animals were housed in individual metabolism cages so that urine and feces could be collected quantitatively.

Throughout this experiment, 24-hr urine samples were collected and alternately assayed for total glucuronic acid and for ethereal sulfate. Total glucuronic acid was determined colorimetrically (Kanabrocki *et al.*, personal communications, 1969), and the ethereal sulfate was analyzed by a turbidimetric method (Bray and Thorpe, 1954).

On day 15 of the experiment the animals were sacrificed, and urine, feces, and fat samples were taken for analysis of radioactivity. Liver samples were homogenized and hepatic enzyme activity was determined in vitro. The enzyme systems investigated were mixed function oxidases [*O*-demethylation of *p*-nitroanisole and oxidative hydrolysis of *O*-ethyl *O*-(4-nitrophenyl) phenylphosphonothioate (EPN) (Kinoshita *et al.*, 1966)], glucuronyl transferase, and the GSH-dependent degradation of γHCH (Ishida, 1968). Glucuronyl transferase activity was determined by a modification of the procedure developed by Kinoshita *et al.* (1966). The final incubation mixture contained 0.2 ml of a solution of uridine-5′-diphosphoglucuronic acid (UDPGA)[5] (0.896 g/100 ml), 0.15 ml of a solution of $MgCl_2$ (1.9046 g/100 ml), 0.25 ml of liver homogenate (1 g/5 ml of 0.25 M sucrose), and 0.2 ml of 0.1 M phosphate buffer containing 5.5 μg of *p*-nitrophenol (pH 7.8). The buffer-substrate solution was prepared by adding 0.5 ml of *p*-nitrophenol solution (0.055 g/100 ml) to 5 ml of the phosphate buffer. The reaction mixtures, in 10-ml beakers, were incubated at 37° C for 30 min under air. The amount of unreacted *p*-nitrophenol was then determined spectrophotometrically as described by Kinoshita.

The weight of the rats, the wet weight of the livers, total liver protein (Gornall *et al.*, 1949), and hepatic cytochrome P-450 content (Alvares *et al.*, 1968) were determined.

[2] The γ-hexachlorocyclohexane-^{14}C, specific activity 37.5 mCi mmole, was obtained from Nuclear-Chicago, Des Plaines, Illinois.

[3] Vitamin A Test Diet was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

[4] Vi-Penta Multi-vitamin Drops were obtained from Hoffmann-La Roche Inc., Nutley, New Jersey.

[5] The ammonium salt of UDPGA was obtained from Sigma Chemical Company, St. Louis, Missouri.

γ-Hexachlorocyclohexane in the adipose tissue was analyzed by electron capture gas chromatography. Lipid content was determined by extracting the adipose tissue with hexane, vaporizing the solvent, and weighing the residue.

The 24-hr feces collection from each rat was homogenized in acetone containing 5% acetic acid (Jensen *et al.*, 1957). This mixture was refluxed and filtered. The residue was again refluxed with fresh solvent and the combined filtrates were concentrated and analyzed for radioactivity. Urine samples were adjusted to pH 7, extracted with benzene, acidified to pH 2, and extracted with benzene, and then ethyl acetate. The urine before and after extraction and all the extracts were analyzed for ^{14}C radioactivity.

Radioactivity of the samples was determined with a Nuclear-Chicago Mark I liquid scintillation counter.

A factorial experiment is designed to statistically evaluate the effects of various factors (here DDT and γHCH) on a measured response. If the effects of the factors are independent, then the main effects of the factors are tested. If the effects of the factors are not independent then the interaction between the factors is tested. The factorial experiment itself provides a test of the assumption of independence.

In addition to the analysis of the main effects or interactions of DDT and γHCH on the measured responses, designed comparison statistical methods were used to aid interpretation of the data (Snedecor, 1956). For example, from a preliminary study it was determined that the effect of γHCH pretreatment on the excretion of glucuronic acid and ethereal sulfate was more pronounced than that of DDT pretreatment. Thus, the following treatment comparisons were analyzed for significant differences: (1) the average excretion of the control rats versus the average excretion of all other rats in the study, (2) the average excretion of the DDT treated rats versus the overall average excretion of the γHCH and DDT + γHCH rats, and (3) the average excretion of the γHCH-treated rats versus the average excretion of the DDT + γHCH-treated animals. Results from the preliminary study also determined the treatment comparisons analyzed in Tables 1 and 2.

Comparisons in this study are considered significantly different at $p < 0.05$.

RESULTS

The effect of the 2-wk pretreatment on rat ability to detoxify γHCH was evaluated by a variety of measured responses: (1) glucuronic acid and ethereal sulfate excretion, (2) excretion of radioactivity, (3) storage of radioactivity, (4) liver weight and liver cytochrome P-450 content, (5) in vitro enzyme activity involved in the oxidative hydrolysis of EPN, the *O*-demethylation of *p*-nitroanisole, the GSH-dependent degradation of γHCH, and the glucuronidation of *p*-nitrophenol.

Glucuronic Acid and Ethereal Sulfate Excretion

Since γHCH metabolites are known to be excreted as glucuronides and sulfates (Grover and Sims, 1965), the excretion of total glucuronic acid (including free and conjugated) and ethereal sulfate was recorded throughout the experiment.

From Fig. 1 it will be observed that within 2 days after treatment began there was a significant DDT × γHCH interaction on the excretion of glucuronic acid. The rats pretreated with DDT + γHCH produced urine containing a significantly higher content

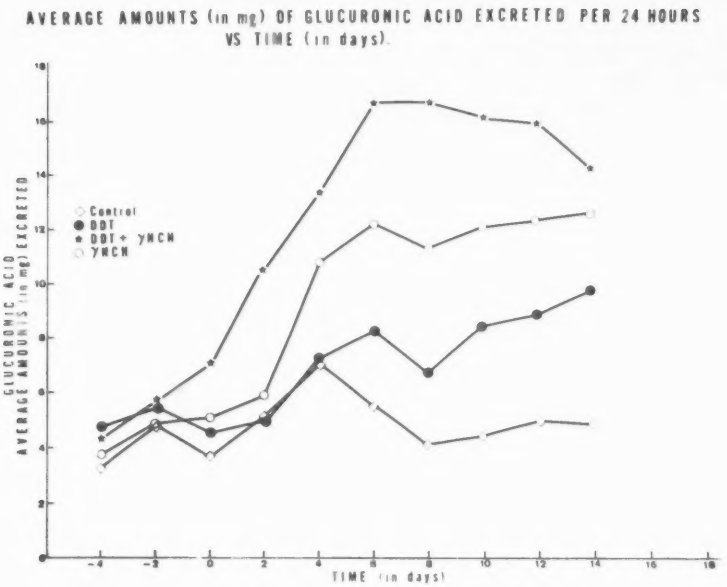

FIG. 1. Average amounts (in mg) of glucuronic acid excreted per 24 hr *vs.* time (in days). Six rats per point.

of glucuronic acid than the other rats in the study. By day 4 of treatment, both the rats fed DDT + γHCH and those fed γHCH were excreting significantly higher quantities of glucuronic acid than the control and DDT-fed rats. After 6 days of treatment, the DDT-treated animals excreted significantly more glucuronic acid than control rats but

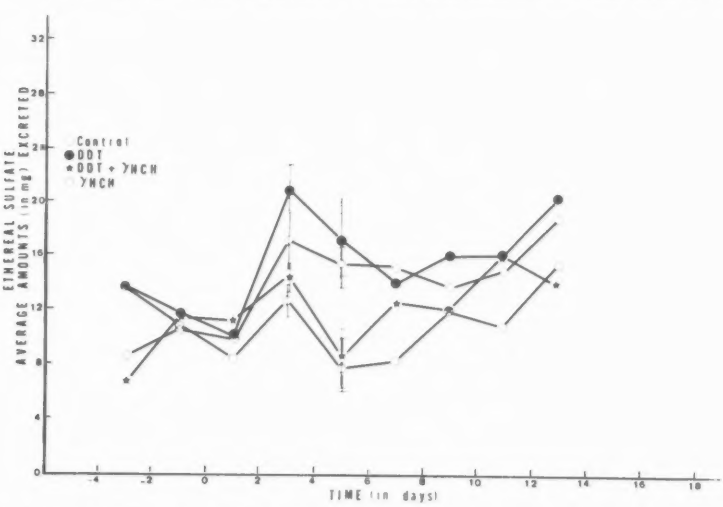

FIG. 2. Average amounts (in mg) of ethereal sulfate excreted per 24 hr *vs.* time (in days). Six rats per point.

significantly less than those receiving γHCH or DDT + γHCH. No further significant changes were noted until the last collection, when the glucuronic acid excreted by the rats treated with DDT + γHCH was no longer significantly higher than that of γHCH-treated rats.

The quantity of ethereal sulfate excreted by the different treatment groups (Fig. 2) was unexpected. It had been anticipated that γHCH would stimulate the excretion of ethereal sulfates, since the trichlorophenol metabolites of γHCH are eliminated as sulfates. Instead, the γHCH-treated rats actually exhibited a significant initial inhibition of this detoxification route. Though no differences were detected 1 day after treatment, a significant decline in the urinary excretion of ethereal sulfates was observed on the third and fifth days of treatment for both the rats treated with γHCH and those treated with DDT + γHCH. Thereafter, the excretion rate of these two groups increased, and the ethereal sulfate content of their urine approached that of the control and DDT-treated animals by the termination of the experiment.

The low ethereal sulfate excretion by rats receiving γHCH or DDT + γHCH may be

TABLE 1

EFFECT OF PRETREATMENT ON THE EXCRETION OF RADIOACTIVITY

Pretreatment		Average measured radioactivity[a]				
DDT (mg)	γHCH (mg)	Benzene Extract I	Benzene Extract II	Ethyl acetate extract	Residual urine	Feces[b] extract
0.0	0.0	2.42 ± 0.32	2.67 ± 0.25	5.35 ± 0.27	1.34 ± 0.09	0.258 ± 0.051
0.0	2.0	4.60 ± 0.53	7.78 ± 0.49	14.6 ± 0.8	2.04 ± 0.09	1.01 ± 0.18
2.0	0.0	10.1 ± 0.9	9.11 ± 0.86	17.4 ± 0.8	2.66 ± 0.26	1.33 ± 0.32
2.0	2.0	9.50 ± 1.11	9.03 ± 0.46	18.8 ± 1.4	2.80 ± 0.28	2.51 ± 0.60

		ANOV[c]				
Source	d.f.	M.S.[d]	M.S.	M.S.	M.S.	M.S.
Control *vs.* treated	1	143[e]	160[e]	604[e]	6.04[e]	693[e]
DDT and DDT + γHCH *vs.* γHCH	1	108[e]	6.56	49.5[e]	1.92[e]	279
DDT + γHCH *vs.* DDT	1	0.982	0.0183	5.57	0.0562	348[e]
Error[f]	20	3.63	1.89	4.90	0.149	62.6
Total[f]	23	14.1	8.90	32.9	0.478	122

[a] Average dpm × 10^{-5} ± SE of the mean.
[b] The average dpm value of the fecal extract is based on 5 rats.
[c] ANOV signifies analysis of variance.
[d] MS signifies mean square.
[e] $p < 0.05$.
[f] The error d.f. is 16 and the total d.f. is 19 for the variance analysis of the feces extract.

the result of product inhibition of sulfokinase. From the results it seems possible that glucuronyl transferase may be more important than sulfokinase in the detoxification of γHCH. Also, the γHCH pretreatment appears to stimulate the formation of glucuronides sooner and to a greater extent than treatment with DDT.

Excretion of ^{14}C-γHCH and Metabolites

Table 1 summarizes the excretion of ^{14}C-γHCH derived material by the 4 treatment groups of rats. Urine was extracted by a succession of solvents as follows: benzene with the urine at pH 7.0 (Benzene Extract I), benzene with the urine at pH 2.0 (Benzene Extract II), and ethyl acetate. Average radioactivity in these extracts, the residual urine, and the extract of the fecal matter are recorded in Table 1.

Total excretion of radioactivity is substantially increased by pretreatment with γHCH, DDT, and DDT + γHCH to 2.5, 3.4, and 3.5 times as much, respectively, as that of the control group. From the results in Table 1, it will be noted that all the urine extracts from the treated animals contain significantly more radioactivity than those of the controls. The rats treated with DDT or DDT + γHCH excrete more radioactivity than the γHCH-treated rats in all fractions except Benzene Extract II and the feces extract.

TABLE 2

DISTRIBUTION OF RADIOACTIVITY

Pretreatment		Average percent of total excreted radioactivity[a]				
DDT (mg)	γHCH (mg)	Benzene Extract I	Benzene Extract II	Ethyl acetate extract	Residual urine	Feces[b] extract
0.0	0.0	19.1 ± 1.7	22.2 ± 0.9	46.0 ± 3.0	11.5 ± 1.3	2.42 ± 0.6
0.0	2.0	14.3 ± 1.4	26.0 ± 1.0	49.5 ± 3.2	7.05 ± 0.5	3.38 ± 0.6
2.0	0.0	25.8 ± 2.1	21.9 ± 2.3	42.4 ± 2.3	6.35 ± 0.8	3.29 ± 0.8
2.0	2.0	20.1 ± 1.8	21.3 ± 0.8	44.3 ± 3.0	6.58 ± 0.3	6.01 ± 1.5

ANOV[c]

Source	d.f.	M.S.[d]	M.S.	M.S.	M.S.	M.S.
Control *vs.* treated	1	340	2.87	1.32	88.6[b]	12.2
DDT and DDT + γHCH *vs.* γHCH	1	253	63.4	126	1.14	5.41
DDT + γHCH *vs.* DDT	1	81.5	0.705	8.46	0.132	18.6
Error	16	15.8	9.87	41	3.42	4.57
Total	19	31.1	11.9	42.1	7.61	5.75

[a] Average percent ± SE of the mean.
[b] $P = 0.05$.
[c] ANOV signifies analysis of variance.
[d] MS signifies mean square.

The only significant difference between the radioactivity excreted by the rats treated with DDT and with DDT + γHCH occurs in the fecal extract. The rats treated with DDT + γHCH excrete significantly more radioactivity in the feces than all other rats in the study.

The mean values in Table 1 indicate substantial differences in the distribution of excreted radioactivity among the treatment groups. Thus, the rats treated with DDT and DDT + γHCH excrete proportionately more radioactivity in Benzene Extract I than the other groups, whereas γHCH-treated rats excrete a higher percentage of the total radioactivity in Benzene Extract II. To test these observations, the amount of radioactivity in each extract was computed as the percentage of the total excreted radioactivity for each rat. The statistical analysis of these values (Table 2) indicates that the γHCH-treated rats do excrete a significantly higher percentage of their total excreted radioactivity in Benzene Extract II than the rats treated with DDT or DDT + γHCH. Results in Table 2 also show that DDT-treated rats excrete a significantly higher proportion of their total radioactivity in Benzene Extract I than do the other animals in the study. Benzene Extract I should contain neutral and weakly polar metabolites whereas Benzene Extract II presumably contains free acid-type compounds.

Enzyme Activity and Metabolic Alterations

The data from this part of the study are summarized in Fig. 3. The metabolic responses measured are liver weight, liver cytochrome P-450 content, and the altered storage of γHCH in body fat. Increases in the first 2 responses frequently accompany

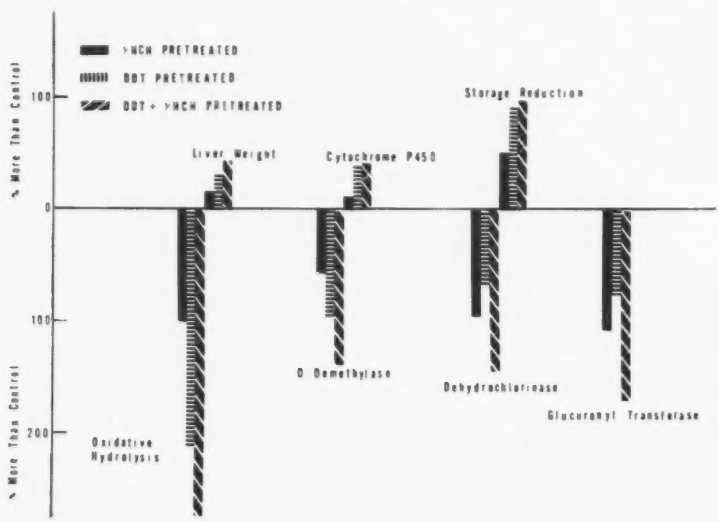

FIG. 3. Pesticide-induced metabolic alterations.

stimulated microsomal enzyme activity; the reduced storage of γHCH is indirect evidence for the increased metabolism of this compound.

Statistical analysis of the data in Fig. 3 shows that treatment with both DDT and

γHCH results in significantly larger livers and significantly less stored radioactivity. However, only treatment with DDT produced a significantly greater cytochromal P-450 content. A highly significant negative correlation ($r = -0.96$) between the amount of stored and excreted radioactivity for all rats in the study suggests that reduced storage results from increased metabolism since GLC analysis indicates that virtually no unaltered lindane is excreted in the urine.

The lower half of Fig. 3 shows the alterations of in vitro enzyme activity. γHCH pretreatment significantly increased the activity of all four of the enzyme systems studied whereas DDT pretreatment produced significant increases only in the oxidative hydrolysis of EPN and the *O*-demethylation of *p*-nitroanisole.

DISCUSSION

Results from this study indicate that pretreatment of rats with DDT, DDT + γHCH, or γHCH alone significantly increases metabolism of γHCH. The conclusion that γHCH induces its own metabolism is based on the statistical evaluation of the accelerated excretion of radioactivity (Table 1), the substantially increased activity of various liver enzyme systems in vitro (Fig. 3), and the 50% reduction in the fat storage of γHCH (Fig. 3). The self-induction mechanism represents a factor of major importance in establishing a steady state relationship between exposure and storage of such pesticides by mammals.

Pretreatment of the rats with either DDT or DDT + γHCH enhances the metabolism of γHCH to a significantly greater extent than γHCH pretreatment. However, the relative importance of various enzyme systems involved in γHCH metabolism seems to depend on the pretreatment. Thus, the microsomal enzymes collectively known as "mixed function oxidases" (Mason, 1957) appear to be primarily responsible for the 3- to 4-fold increase in γHCH metabolism by DDT-pretreated rats. On the other hand, the mechanism of self-induction exhibited by γHCH-pretreated rats involves an enhanced GSH-dependent degradation of γHCH and stimulated glucuronyl transferase activity as well as increased mixed function oxidase activity. The combined DDT + γHCH pretreatment demonstrates the greatest in vitro enzyme activity of all and possibly utilizes 2 different metabolic mechanisms.

Evidence for the existence of different induction mechanisms includes the different distribution of ^{14}C-γHCH derived compounds, apparently determined by the pretreatment. γHCH-pretreated animals excrete a significantly greater proportion of free acid-type metabolites than the rats of the other treatment groups. On the other hand, the DDT-pretreated rats produce significantly higher percentages of neutral and weakly polar γHCH metabolites. In addition, there is a significant correlation between the excretion of neutral and weakly polar metabolites and the cytochrome P-450 content of the liver ($r = 0.58$). Cytochrome P-450 has been implicated as the terminal oxidase in microsomal enzyme systems which hydroxylate drugs and steroids (Remmer and Merker, 1965). Thus, hydroxylation may be more important in the metabolism of γHCH by DDT-pretreated rats than by γHCH-pretreated animals. The excellent correlation between excreted radioactivity and the in vitro activity of the mixed function oxidases ($r = 0.87$ for oxidative hydrolysis of EPN versus excreted radioactivity and $r = 0.76$ for *O*-demethylase of *p*-nitroanisole versus excreted radioactivity) lends additional support to this proposal.

Enzyme-inducing agents are usually assigned to one of 3 rather broad categories: (1) anabolic steroids, (2) polycyclic hydrocarbons, (3) barbiturates and miscellaneous drugs including organochlorine insecticides (Mullen *et al.*, 1966). The first 2 categories are quite specific and stimulate only a few microsomal enzymes. Agents in the third group are believed to bring about a generalized nonspecific induction of microsomal enzymes through similar mechanisms. A few workers have already observed differences in the stimulation of selected enzyme activities by agents from the third category. Lange (1967) found that phenobarbital stimulated dealkylation of *N*-ethylaniline but DDT did not. Kato and co-workers have described differences in the hydroxylation of pentobarbital or hexobarbital from that of aniline or zoxazolamine when various inducing agents, inhibitors, and experimental conditions were employed. They also detected differences in the *N*-demethylation of aminopyrine and *N*-methylaniline (Kato *et al.*, 1969). Street (1968) questioned the mechanistic similarity of DDT, heptabarbital, 3-methylcholanthrene, and chlordane in stimulating dieldrin metabolism and suggested the possibility of rather selective responses to these stimulating agents. The results of the present study also tend to disagree with the assumption that organochlorine insecticides induce microsomal enzymes through similar nonspecific mechanisms.

Koransky and co-workers (1964) demonstrated enhanced γHCH metabolism in rats pretreated with phenobarbital. The phenobarbital pretreatment resulted in a great stimulation of the oxidative transformation of γHCH and a simultaneous decline in the dehydrochlorination of the insecticide. Nakatsugawa *et al.* (1965) have suggested that an oxidation of γ-2,3,4,5,6-pentachlorocyclohex-1-ene (PCCH) may be involved in the metabolism of γHCH. PCCH is presently regarded as an early intermediate in γHCH metabolism (Grover and Sims, 1965). The data of the present experiment in conjunction with those of previous workers lead to speculation that the highly enhanced γHCH metabolism exhibited by DDT-pretreated rats is the result of an altered metabolic pathway involving stimulated oxidation at an early stage.

Alterations in the biochemical and metabolic functions in mammals caused by interactions between various combinations of pesticides, drugs, and other environmental chemicals are becoming increasingly important (DuBois, 1969). In fact, one recent report indicates the existence of a negative correlation between life expectancy in the rat and the levels of hepatic enzyme activity (Ross, 1969). Methods must be developed for screening synthetic chemicals to discover potential inducing agents and to evaluate their effects on hepatic enzyme activity. Currently, time-consuming studies of in vitro enzyme levels are being employed for this purpose. Another possible solution could involve repeated pretreatment of experimental animals with the compound of interest followed by a single administration of a "model compound." Metabolism of the model compound would ideally involve numerous enzyme systems, the induction of which could be determined by gas chromatographic examination of the metabolite distribution. γ-Hexachlorocyclohexane may be such a compound, since its metabolism involves dehydrochlorinase, glutathione-*S*-aryltransferase, glucuronyl transferase, sulfotransferase, epoxidase, and various other mixed function oxidases (Grover and Sims, 1965; Nakatsugawa *et al.*, 1965). Unfortunately all the metabolites of γHCH have not been characterized, but work in this area is currently underway at this and other laboratories.

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Storage and Excretion of Dieldrin and Related Compounds

Effect of Occupational Exposure

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IT HAS been shown that dieldrin is present in the body fat,[1-13] blood,[12,14-17] and urine[18] of most people in the general population of this country,[1,3,4,7,8,16-18] the United Kingdom, [2,6,12,14,15] India,[5] Denmark,[13] and Italy.[11] (The word dieldrin is used in this paper to designate the single compound [1,2,3,4,10,10-hexachloro-6, 7-epoxy-1,4,4a,5, 6,7,8,8a-octahydro-1, 4-*endo-exo*-5, 8-dimethanonaphthalene] sometimes called HEOD. The term "technical dieldrin" designates the commercial product consisting of a mixture of compounds containing not less than 85% of dieldrin). Dieldrin probably occurs too in the people of some other countries where measurements of it have not been made or at least have not been reported. It is also known that much higher concentrations occur in persons who are exposed occupationally to dieldrin or to aldrin[15,16,19,20] and that even higher concentrations occur in persons who are poisoned by one of the compounds through accident or suicide.[21,22]

Aldrin, too, has been found in human tissue,[8,11,16,22] but this compound is detected less often than dieldrin largely because—as pointed out by Barnes[23] and confirmed by others—absorbed aldrin is converted to dieldrin in the body. (The word aldrin is used in this paper to designate the single compound [1,2,3,4,10,10-hexachloro-1, 4,4a,5,8,8a-hexahydro-1, 4-*endo-exo*-5, 8-dimethanonaphthalene] sometimes called HHDN. The term "technical aldrin" designates the commercial product consisting of a mixture of compounds containing not less than 95% of aldrin).

In spite of the rather considerable work already reported on the storage and excretion of dieldrin and related materials in man, there is only limited information[15] on how these parameters are related to the intensity and duration of occupational exposure. Under these circumstances, we were pleased when David R. Metcalf, MD, requested the analysis of tissues in connection with electroencephalographic studies he had under way and will report separately. Through his help, we obtained part of the samples in sets so that a comparison could be made between the concentrations of dieldrin-related materials in fat, blood, and urine.

This paper reports the analytical results and relates them to detailed individual work records maintained by the company where the exposed men are employed.

Methods

The factory where the men who contributed samples are employed manufactures dieldrin, aldrin, endrin, a nematocide, and certain organic phosphorus compounds. Detailed work records have been maintained by IBM tabulation since 1957, but some of the workers have been doing the same kind of work for a longer period. The complete work record for each extensively exposed employee lists separately the number of hours of his exposure to the pesticides and their precursors. In addition, each work record lists the number of hours spent in shipping operations, the number of hours of work not involving exposure to chemicals, the number of hours of sick leave, and the worker's classification—for example, operator or pipe fitter.

A total of 71 men participated in the study

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Table 1.—*Concentration of Chlorinated Hydrocarbon Pesticides and Their Metabolites*

	Kind of Sample		
	Plasma	Fat	Urine
No. of samples	114	32	14
Concentration of *p, p'*-DDT			
Range (ppm)	<0.0006—0.0383	<0.26—5.39	<0.0005—0.0138
Mean ± SE (ppm)	0.0075 ± 0.0005	1.15 ± 0.19	0.0063 ± 0.0008
Concentration of *o, p'*-DDT			
Range (ppm)	<0.0001—0.0084	all <1.00	<0.0002—0.0026
Mean ± SE (ppm)	0.0010 ± 0.0001		0.0013 ± 0.0003
Concentration of *p, p'*-DDE			
Range (ppm)	0.0022—0.0526	0.021—14.33	0.0019—0.0289
Mean ± SE (ppm)	0.0167 ± 0.0009	4.87 ± 0.56	0.0143 ± 0.0017
Concentration of *o, p'*-DDE			
Range (ppm)	<0.0001—0.0080	>0.45—1.15	<0.0002—0.0007
Mean ± SE (ppm)	0.0007 ± 0.0001	0.46 ± 0.04	0.00008 ± 0.00006
Concentration of *p, p'*-DDD			
Range (ppm)	<0.0001—0.0139	<0.21—0.63	0.0013—0.0092
Mean ± SE (ppm)	0.0045 ± 0.0004	0.07 ± 0.03	0.0045 ± 0.0006
Concentration of α-BHC			
Range (ppm)	<0.0001—0.0006	all <0.01	<0.0002—0.0033
Mean ± SE (ppm)	0.00002 ± 0.000009		0.0010 ± 0.0003
Concentration of β-BHC			
Range (ppm)	<0.0001—0.0363	<0.01—1.18	<0.0004—0.0012
Mean ± SE (ppm)	0.0018 ± 0.0002	0.46 ± 0.06	0.0001 ± 0.00009
Concentration of γ-BHC			
Range (ppm)	<0.0001—0.0031	all <0.01	<0.0002—0.0004
Mean ± SE (ppm)	0.0003 ± 0.00006		0.00016 ± 0.00004
Concentration of heptachlor epoxide			
Range (ppm)	<0.0001—0.0155	<0.16—0.64	all <0.0002
Mean ± SE (ppm)	0.0010 ± 0.0002	0.11 ± 0.03	
Concentration of aldrin			
Range (ppm)	<0.0001—0.0039	all <0.01	all <0.0002
Mean ± SE (ppm)	0.0006 ± 0.00007		
Concentration of dieldrin			
Range (ppm)	<0.0007—0.1370	0.60—31.96	0.0013—0.0660
Mean ± SE (ppm)	0.0185 ± 0.0019	5.67 ± 1.11	0.0242 ± 0.0063
Concentration of dieldrin in the general population*			
Range (ppm)	0.0007—0.0037	0.03—1.15	0.0005—0.0014
Mean ± SE (ppm)	0.0019 ± 0.0003	0.29 ± 0.05	0.0008 ± 0.0002

* These published values for the general population were determined in the same laboratory and are directly comparable to the values for dieldrin in workers. See reference 16 on plasma, reference 7 on fat, and reference 18 on urine.

reported in this paper. Complete work records dating from 1957 (or from the worker's first employment if later than 1957) were supplied for 49 men. They had been employed at the same kind of work for 1 to 19 or an average of 12 years. It was stated that 14 other men had minimal to moderate exposure in connection with their duties as foremen, painters, carpenters, engineers, inspectors, and laboratory workers. The remaining eight were clerical workers.

Chemical analyses of value for this paper were completed for 114 samples of plasma from 71 men, 32 samples of fat from 32 men, and 14 samples of urine from 14 men. A considerable number of additional urine samples collected during the same period were used in a study of analytical methods reported separately.

Blood was collected by venipuncture. Potassium oxalate was used as an anticoagulant since some other anticoagulants, especially heparin, interfere with the measurement of chlorinated hydrocarbon insecticides by electron-capture gas-liquid chromatography without cleanup. The plasma was separated and kept under refrigeration. Fat samples weighing 65.2 to 180 mg were taken by surgical biopsy and preserved by freezing. Voided samples of urine were preserved with a small amount of formaldehyde.

The samples of plasma and fat packed in dry ice were shipped to the analytical laboratory by air.

All the samples were analyzed by gas-liquid chromatography using an electron-capture detector. The method of Dale et al[16] was used for plasma, that of Read and McKinley[24] for fat, and that of Cueto and Biros[18] for urine.

Table 2.—*Concentration of and Exposure to Dieldrin, Sick Time, and Correlations Among These Variables*

	Kind of Sample		
	Plasma	Fat	Urine
No. of samples	28*	28*	9†
Concentration of dieldrin			
Range (ppm)	0.0012 — 0.1370	0.60 — 31.96	0.0014 — 0.0660
Mean ± SE (ppm)	0.0247 ± 0.0060	6.12 ± 1.24	0.0284 ± 0.0087
Total exposure to dieldrin			
Range (hrs)	27 — 14,066	27 — 14,066	58 — 11,175
Mean (hrs)	2,624	2,624	2,569
Exposure to dieldrin in preceding year			
Range (hrs)	0 — 1,448	0 — 1,448	0 — 344
Mean (hrs)	118	118	45
Average sick leave per year			
Range (hrs)	0 — 136	0 — 136	5 — 167
Mean (hrs)	46	46	59
Correlation (r-value) for concentrations of dieldrin in:			
plasma	...	+0.857	+0.907
fat	+0.857	...	+0.797
Correlation (r-value) for concentration of dieldrin in tissue with:			
total hours exposure to dieldrin	+0.416	+0.483	+0.470
hours of exposure to dieldrin in preceding year	+0.081	+0.043	+0.466
average hours sick leave per year	−0.0198	+0.031	+0.178

* Each of 28 men contributed a sample of blood and a sample of fat on the same day.
† Of the 28 men, each of 9 contributed a urine sample, but not on the same day as the other samples.

Results

Analytical Findings.—The analytical results for all available samples are summarized in Table 1. Eleven isomers or metabolites representing six insecticides (DDT, DDD, BHC, dieldrin, aldrin, and heptachlor) were detected in plasma, but not all were found in every sample. In fact, *p,p'*-DDE was the only compound detected in every sample of plasma; it was found in every sample of fat and urine also. The average concentration of dieldrin in the plasma of workers—even counting the clerical help—was over nine times greater than that for the general population (Table 1). Aldrin was also found in many of the plasma samples although it cannot be detected at all by present methods in the blood of people without special exposure. Three compounds—*p,p'*-DDT, β-BHC, and heptachlor epoxide—were found in at least one sample at slightly higher concentrations than we have previously found them in the general population,[16] but the differences are small, and the mean concentration for these three compounds in the workers was actually smaller than has been found earlier for a population without any special exposure. For each of the remaining isomers or metabolites, the highest concentration found was less than the maximum previously found in people with no special exposure.

Only seven isomers or metabolites representing five insecticides were found in the fat samples; no aldrin was detected. The average storage of dieldrin in the fat of the workers was over 19 times that in the general population. The concentration of the other compounds, except *o,p'*-DDE, did not exceed the concentration found in the general population. The difference for *o,p'*-DDE was small.

Only nine isomers or metabolites representing only four insecticides were found in urine. Aldrin was not detected in any sample, but the concentrations of dieldrin-related material averaged about 30 times higher than those found by the same method in the urine of men with no special exposure to pesticides.

It is noteworthy that no endrin was found in any sample even though, with the exception of one unexposed person, those with a complete work history had been exposed to it for an average of 2,106 hours. In view of the size of the samples available and the sensitivity of the method, the concentrations of

endrin were < 0.0030 ppm in plasma, < 0.03 ppm in fat, and < 0.0016 ppm in urine. Although no endrin was detected in these individuals, we have measured it without difficulty in the serum and urine of persons who ate the compound by accident.[25]

Correlation of the Concentrations of Dieldrin in Different Kinds of Samples.—In a statistical investigation of a number of relationships, particular attention was given to 28 pairs of samples of plasma and fat collected from men with complete work histories. Each man gave both of his samples on the same day, but different men were sampled on different days. Attention was also given to nine samples of urine contributed by nine of the same men but not on the same day as the other samples. Analytical data on these samples and various correlations among them are shown in Table 2.

Table 3.—*Relation of the Concentration of Dieldrin in Plasma and Urine to Total Exposure to Technical Dieldrin

	Kind of Sample	
	Plasma	Urine
No. of samples	49	11
Concentration of dieldrin		
Range (ppm)	0.0012—0.1083	0.0014—0.0660
Mean ± SE (ppm)	0.0206 ± 0.0029	0.0294 ± 0.0072
Total hours exposure to dieldrin		
Range (hrs)	11—14,066	58—11,175
Mean ± SE (hrs)	2,066 ± 452	2,361 ± 957
Correlation (*r*-value) for concentration of dieldrin with:		
total hours exposure to dieldrin	+0.605	+0.406

* In instances in which a worker gave more than one sample (blood or urine), only a single sample was considered in this table.

Table 4.—*Concentration of Aldrin in Plasma

	Plasma
No. of samples	49
Concentration of aldrin	
Range (ppm)	0.0001 — 0.0032
Mean ± SE (ppm)	0.0007 ± 0.0001
Total hours exposure to aldrin	
Range (hrs)	0 — 4,972
Mean ± SE (hrs)	1,102 ± 170
Exposure to aldrin in preceding year	
Range (hrs)	0 — 1,336
Mean ± SE (hrs)	184 ± 37
Correlation (*r*-value) for concentration of aldrin with:	
total hours exposure	+0.18
exposure in preceding year	+0.22

* Relation to total exposure to the technical material, and also to exposure during the preceding year only.

The coefficients of correlation (*r*-values) for the concentrations of dieldrin in plasma, fat, and urine were +0.8 or greater—a high correlation for biological samples.

The average concentration of dieldrin in fat was 247 times greater than its average concentration in plasma. The factor was similar for fat and urine.

Relation of the Concentration of Dieldrin-Related Materials in Samples to Work History.—The relation of the concentration of dieldrin-related materials to the work history was explored for the samples referred to in Table 2. The *r*-value was +0.4 or slightly greater when the concentration of dieldrin in plasma, fat, or urine, respectively, was compared to total hours of exposure to the same compound.

The frequently poor correlation between concentration of dieldrin and exposure during the preceding year was explained to a large extent by the finding of substantial concentrations of dieldrin in samples from men who had no exposure to the compound during the year preceding the sample, but who had had extensive exposure earlier. Since the presence of dieldrin in samples was more closely correlated with total exposure than with exposure during the last year, all further comparisons for dieldrin were made in terms of total exposure.

There was no meaningful correlation between the occurrence of dieldrin in samples and average sick leave per year. During the period for which records were available (1957 through the end of the study in 1966), no time was missed because of pesticides.

Comparisons similar to those discussed above were made for all situations. The presence of analytical results for plasma or urine and the availability of a complete work history made this possible (Tables 3 to 5). Coefficients of correlation of +0.4 or more were obtained for comparisons of (1) dieldrin in plasma against total exposure to dieldrin (2) dieldrin-related material in urine against total exposure to dieldrin, and (3)

Table 5.—Concentration of Aldrin Plus Dieldrin in Plasma*

	Plasma
No. of samples	48
Concentration of aldrin plus dieldrin	
Range (ppm)	0.0012 — 0.1083
Mean ± SE (ppm)	0.0213 ± 0.0029
Total exposure to aldrin plus dieldrin	
Range (hrs)	76 — 15,522
Mean ± SE (hrs)	3,168 ± 513
Correlation (r-value) for concentration of aldrin plus dieldrin with:	
total hours exposure to aldrin plus dieldrin	+0.591

* Relation to total exposure to both technical compounds.

sum of dieldrin and aldrin in plasma against total exposure to both compounds.

Probably because of the rather rapid metabolism of aldrin to dieldrin, the correlation was poor for aldrin in plasma against exposure to technical aldrin, whether judged on the basis of total hours of exposure or hours of exposure during the preceding year.

The various comparisons mentioned above were confined to cases with a complete work record, ie, one enumerating the hours of exposure to each insecticide or precursor for each work period. Additional comparisons were made in which it was assumed that a record of moderate, minimal, or no exposure was equivalent to zero hours of exposure. This permitted consideration of all of the men from whom samples were obtained. Comparison of values in Table 6 with relevant ones in Tables 2 and 3 indicates—perhaps as might be expected—that the inclusion of people with a wider range of exposure produced a slight increase in the coefficients of correlation relating degree of exposure to dieldrin with its concentration in tissue and excreta.

In 38 instances, two or more samples of blood were collected from the same man at different times. When the concentration of dieldrin in plasma was plotted against the date of collection, the curves indicated no common trend in dieldrin content. The concentration decreased with time as often as it increased. The evidence is not adequate but suggests that, insofar as their exposure was constant, the workers under study had reached a steady state of dieldrin storage.

The concentration of dieldrin in plasma was compared with the job classifications of the men. The results are shown in Table 7. The separation of different kinds of jobs into groups was based entirely on the judgment of plant management. There was a small but statistically insignificant difference ($P > 0.4$) between the values for group 1 (mainly pipefitters, shippers, and helpers) and group 2 (operators, operations foremen, and pipefitting foremen). The plasma values for group 3 (painters, carpenters, instrument men, machinists, engineering foremen, safety inspectors, and laboratory workers) were distinctly less than those for groups 1 and 2 combined ($P < 0.01$) and distinctly greater than those for the general population ($P < 0.001$). The plasma values for group 4 (clerical workers) were not significantly different from those for group 3 ($P > 0.1$), and although the values for group 4 were slightly greater than those for the general population as reported by Dale et al[16] the

Table 6.—Dieldrin in Plasma, Fat, and Urine*

	Kind of Sample		
	Plasma	Fat	Urine
No. of samples	71	32	14
Concentration of dieldrin			
Range (ppm)	<0.0007 — 0.1083	0.60 — 31.96	0.0013 — 0.0660
Mean ± SE (ppm)	0.0162 ± 0.0022	5.67 ± 1.11	0.0242 ± 0.0063
Total hours exposure to dieldrin			
Range (hrs)	0 — 14,066	0 — 14,066	0 — 11,175
Mean ± SE (hrs)	1,426 ± 331	2,296 ± 655	1,854 ± 791
Correlation (r-value) for concentration of dieldrin with:			
total hours exposure to dieldrin	+0.633	+0.507	+0.492

* Relation to total exposure to technical dieldrin. Unlike earlier tables, this one includes values from men with no recorded exposure to dieldrin, although they were employed in a plant where it was made. In instances in which a worker gave more than one kind of sample (blood or urine) only a single sample was considered in this table.

Table 7.—Concentration of Dieldrin in the Plasma of Men With Different Degrees of Occupational Exposure

Group		Dieldrin in Plasma		Total Exposure*		
				No. of Records		
No.	Composition	No. of Samples	Range Mean ± SE (ppm)	De-tailed	No hourly data	Range Mean ± SE (hrs)
1.	Pipefitters, shippers, and helpers	20	0.0012 — 0.0458 0.0233 ± 0.0039	20	0	58 — 7,462 1,417 ± 387
2.	Operators, foremen	26	0.0032 — 0.1083 0.0189 ± 0.0045	21	5†	27 — 14,066 3,165 ± 944
3.	Painters, carpenters, engineers, inspectors, laboratory workers	17	0.0013 — 0.0215 0.0098 ± 0.0016	8	9†	11 — 1,570 802 ± 188
4.	Clerical workers	8	<0.0007 — 0.0255 0.0054 ± 0.0030	0	8‡	
	Total	71		49	22	

* Range and mean based on detailed, hourly data.
† Total of 14 with minimal to moderate exposure.
‡ There were eight men stated to have no exposure.

difference did not reach statistical significance ($P > 0.1$).

It may be seen from Table 7 that nearly all of the complete work histories available were for men in groups 1 and 2. As pointed out earlier, there was a correlation of +0.6 between concentration of dieldrin in plasma and total hours of exposure.

Comment

It is noteworthy that there was no meaningful correlation between the use of sick leave and the concentration of dieldrin and related compounds in samples measured in this study. However, other studies of dieldrin as well as the general principles of toxicology indicate that the degree of toxicity is correlated with tissue levels. So far as whole blood is concerned, Brown et al[15] suggested that the threshold of injury corresponds to levels of 0.15 to 0.20 ppm. Presumably, a slightly higher threshold would apply to plasma or serum since they contain slightly higher concentrations of dieldrin than corresponding samples of whole blood contain.[16] Initial levels of 0.279 ppm for dieldrin and 0.036 ppm for aldrin were measured in this laboratory in the plasma of a man who survived poisoning by aldrin taken with suicidal intent. Thus, our findings are not inconsistent with the current estimates of the dangerous blood level for dieldrin.

The results reported in this paper indicate that samples of urine might be valuable for monitoring the absorption of aldrin and dieldrin by persons who are occupationally exposed to them. However, before this use becomes fully practical, there will have to be further research to learn what concentration of dieldrin-related material in urine corresponds to a dangerous level of absorption of aldrin or dieldrin, or both.

Judging from the analytical results reported in this paper, the three kinds of samples would be of about equal value as quantitative measures of absorption of dieldrin. The correlation between them was high for dieldrin. Undoubtedly, more metabolites or isomers of various other insecticides could have been detected in fat if large samples had been taken. The fact that aldrin was found in plasma but not in fat or urine is a reason to use this kind of sample for routine monitoring of men with special exposure to aldrin. Whether this advantage of blood samples is sufficient to compensate for the ease of getting urine samples is not clear.

It is important to remember that not only dieldrin but also aldrin and endrin or their derivatives can be measured in human blood and urine provided exposure is sufficient. Analysis of samples of either serum or urine or both would appear to be an ideal way of monitoring the exposure of workers to these compounds. It should be possible to eliminate any possibility of injury from repeated exposure by limiting opportunity for absorption.

Although the present study was carried out with plasma, other work in this laboratory[16] indicates that serum is equally satisfactory for the analysis of chlorinated hydrocarbon insecticides. Serum is slightly more convenient than plasma to collect, and some

Table 8.—Estimation of the Daily Intake of Dieldrin for Men Who Contributed Matched Samples of Plasma, Fat, and Urine

	Kind of Sample		
	Plasma	Fat	Urine
No. of samples	9	9	9
Total hours exposure to dieldrin			
Range (hr)	58—11,175	58—11,175	58—11,175
Mean ± SE (hr)	2562 ± 1167	2562 ± 1167	2562 ± 1167
Concentration of dieldrin*			
Range (ppm)	.0068—.1370	2.61—31.96	.0014—.0660
Mean ± SE (ppm)	.0411 ± .0164	9.48 ± 3.33	.0284 ± .0088
Estimated intake of dieldrin†			
(mg/man/day)	1.00	1.10	0.72

* No aldrin was detected in any of these samples.
† Based on mean concentration for each kind of sample.

anticoagulants, especially heparin, interfere with the analysis of chlorinated hydrocarbon insecticides by electron-capture, gas-liquid chromatography without cleanup. We conclude that, if only one blood fraction is to be used, serum is the ideal one for measuring the concentration of chlorinated hydrocarbon insecticides in blood.

In the present study the average concentration of dieldrin in fat was 247 times greater than that found in blood. This factor was essentially the same whether based on all appropriate pairs of samples or only on those showing high or low concentrations of dieldrin. The factor of 247 may be compared to one of 197 at 250 days (all dosage groups) or 138 at 445 days (all dosage groups) as calculated in the same way from data by Hunter and Robinson.[25]

If, on the other hand, the factors are calculated as Hunter and Robinson did (ie, from a significant regression of fat on blood), then the result is 179 for our data and 156 for theirs. The order of magnitude of the factor is the same according to both sets of measurements; the similarity is certainly more important than any small, real difference that may exist.

Rather high coefficients of correlation were obtained for the concentration of dieldrin in various samples compared to the total hours of exposure. In some instances significant differences were also found between the concentration of dieldrin in plasma of men with different job classifications. The available data are not adequate to determine how much of the difference in absorption of insecticides associated with different kinds of jobs is explained by duration of exposure and how much is associated with real differences in the average intensity of exposure per hour of the different kinds of work. It is probable that an even higher correlation could be demonstrated between exposure to dieldrin and its concentration in samples if an objective way could be found to take into account simultaneously both duration and intensity of exposure.

The absolute efficiency of measuring dieldrin-related material in human urine is not known nor is it known what proportion of the total excretion is by that route. Cueto and Biros[18] found dieldrin-related material at an average concentration of 0.001 ppm in the urine of men and women in the general population. Assuming a daily urinary volume of 1.5 liters, this would account for 1.5μg/man/day, or about 6% of the 24 to 25μg/man/day, which Hunter and Robinson[26] estimated to be the total rate of intake of dieldrin by the general population. In any event the amount recovered from the urine constitutes a minimal measure of excretion and, therefore, of absorption. Assuming a daily urinary volume of 1.5 liters, the average minimal urinary excretion of dieldrin by men who submitted samples of urine as well as samples of plasma and fat in the present study was 0.043 mg/man/day. If this rate constitutes 6% of total average intake, then the latter was approximately 0.7 mg/man/day.

The total average daily intake of dieldrin for the same men who submitted urine samples can be estimated in two other ways, ie, from the average concentrations of dieldrin in their plasma and fat. This is possible because of the studies of Hunter and Robinson[25] in which known doses of dieldrin were given to volunteers until they approached a steady state of storage. Based on the relationships they found between dosage and concentrations in blood and fat, respectively, the estimated intake of the men mentioned above is 1.0 or 1.1 mg/man/day. The estimates are based on analytical values shown in Table 8. It may be seen by comparing these storage values for dieldrin with those for the

entire group of men studied (Table 1) that the estimate of intake involved only men with substantial exposure to dieldrin. These estimates of the daily intake of dieldrin by workers (0.72 to 1.1 mg/man/day) may be compared to the highest dosage of dieldrin given to volunteers by Hunter and Robinson, ie, 0.21 mg/man/day.

Summary

Chlorinated hydrocarbon insecticides and their metabolites were measured by electron-capture, gas-liquid chromatography in 114 sample of plasma, 32 samples of fat, and 14 samples of urine from 71 men who manufacture aldrin, dieldrin, and endrin. A rather high coefficient of correlation ($r = +0.8$ or more) was found for the concentration of dieldrin in different kinds of samples from the same person. The concentration of dieldrin in samples showed a good correlation with total duration of exposure ($r = +0.41$ to $+0.63$) or with the intensity of exposure as indicated by the kind of work. The levels of aldrin and dieldrin found in the exposed workers were significantly greater than those in the general population.

In the present study, no significant relation existed between the amount of dieldrin in samples and the use of sick leave. However, other studies in this and other laboratories have shown that higher levels of dieldrin are associated with poisoning. Since the concentration of dieldrin in samples is correlated both with exposure and biological effect, measurement of the compound offers a valuable way of monitoring absorption of technical aldrin and dieldrin by workers and, thus, preventing their excessive exposure. Partly for reasons of convenience, serum is now the best kind of sample for this purpose. Because of their ease of collection, urine samples may eventually become preferable provided more can be learned about the relation of urinary levels of dieldrin-related material to poisoning.

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